UNIVERSIDADE FEDERAL DO PARANÁ

DAIANI CRISTINA SAVI

GÊNERO *Microbispora*: RECLASSIFICAÇÃO FILOGENÉTICA, BIOPROSPECÇÃO E IDENTIFICAÇÃO DE METABÓLITOS SECUNDÁRIOS

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia, Parasitologia e Patologia, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito parcial à obtenção de título de Doutora em Microbiologia.

Orientadora: Profª Drª Chirlei Glienke

Co-Orientadores: Drª Josiane Gomes Figueiredo e Dr Jurgen Rohr

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TERMO DE APROVAÇÃO

"GÊNERO *Microbispora*: RECLASSIFICAÇÃO FILOGENÉTICA, BIOPROSPECÇÃO E IDENTIFICAÇÃO DE METABÓLITOS SECUNDÁRIOS"

por

DAIANI CRISTINA SAVI

Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, pela Comissão formada pelos professores:

Drª. Josiane Aparecida Gomes Figueiredo – Presidente

Profª. Drª. Yvelise Maria Possiede

Profª. Drª. Lygia Vitória Galli-Terasawa

Profª. Drª. Beatriz H. L. N. Sales Maia

Profª. Drª. Vanessa Kava-Cordeiro

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“No problem can be solved from the same level of consciousness that created it”

Albert Einstein

RESUMO

A ioprospecção de microrganismos hoje é a fonte mais promissora para obtenção de novos compostos bioativos. Actinomicetos do solo pertencentes ao gênero *Streptomyces*, produzem uma vasta gama de compostos amplamente utilizados pela industría farmacêutica e agronômica. Porém, devido ao grande número de pesquisas envolvendo estes microrganismos a probablidade da descoberta de novos compostos a partir de linhagens isoladas do solo torna-se cada vez menos provável, levando a exploração de actinomicetos de outras fontes naturais, como por exemplo, isolados de plantas medicinais. Neste contexto, o presente trabalho objetivou o isolamento, bioprospecção, e a identificação de actinomicetos endofíticos da planta medicinal *Vochysia divergens*. O trabalho foi dividido em 3 capítulos voltados a bioprospecção e avaliação da atividade biológica dos isolados endofíticos - principalmente pertencentes ao gênero *Microbispora* - seguido de identificação dos compostos produzidos pela linhagem mais promissora e posterior estudo filogenético dos isolados pertencentes ao gênero *Microbispora*. O primeiro capítulo, intitulado **"Antitumor, antioxidant and antibacterial activities of secondary metabolites extracted by endophytic actinomycetes isolated from *Vochysia divergens*"**, explorou o isolamento e biodiversidade de actinomicetos isolados da planta *Vochysia divergens*, bem como a capacidade de seis isolados em produzir compostos com atividade antibacteriana, antioxidante e antitumoral. O segundo capítulo, ***"Microbispora* sp. LGMB259 Endophytic Actinomycete Isolated from *Vochysia divergens* (Pantanal, Brazil) Producing *β*-Carbolines and Indoles with Biological Activity"** objetivou o isolamento e identificação de compostos produzidos pela linhagem de *Microbispora* sp. LGMB259. No trabalho foram identificados sete compostos, quatro β-carbolinas e três indóis, sendo que o composto 1-vinil-*β*-carbolina-3-ácido carboxílico foi responsável pela atividade biológica desta linhagem, o qual apresentou atividade antibacteriana, antifúngica e citotóxica. O último capítulo, intitulado **"Multilocus Sequence Analysis of the Genus *Microbispora*"** baseou-se em análise multigênica utilizando os genes 16S rRNA, 23S rRNA, *gyr*B e *rpo*B para o estudo filogenético dentro do gênero *Microbispora*. As análises realizadas sugerem que as espécies *M. amethystogenes, M. chromogenes, M. karnatakensis, M. parva, M. aerata, M. thermodiastatica* e *M. thermorosea* são espécies distintas da espécie *M. rosea*; diferentemente do proposto na literatura por Miyadoh et al. (1990). *M. aerata* e *M. thermodiastica* compartilharam elevada similaridade genética e provavelmente pertencem a mesma espécie, assim como *M. indica* e *M. rosea*. As linhagens endofíticas pertencentes aos clusters *Microbispora* sp. 1, *Microbispora* sp. 2 e *Microbispora* sp. 3 são diferentes das espécies de *Microbispora* previamente descritas, sendo que mais estudos são necessários para a descrição das mesmas como novas espécies. Foi proposto também a análise concatenada dos genes *gyr*B-*rpo*B como uma alternativa à técnica de hibridização de DNA para a identificação de espécies dentro do gênero *Microbispora*, e o valor de 98,0% como o valor de homologia mínimo para classificar os isolados de *Microbispora* como pertencentes a mesma espécie.

Palavras-chave: *Vochysia divergens* – Gênero *Microbispora* – metabólitos secundários – β-carbolinas – reclassificação filogenética

ABSTRACT

The Bioprospecting of Microorganisms is the most promising source for new bioactive compounds. Actinomycetes - mainly that belonging to the genus *Streptomyces* - isolated from soil produced a wide range of bioactive compounds widely used by the pharmaceutical and agronomic industries. However, due to the large number of research involving these microorganisms the probablidade of discovery novel compounds isolated from soil strains is increasingly less, leading to exploration of actinomycetes isolated from others natural sources such as, endophytic isolated by medicial plants. In this context, our objectives were the isolation, bioprospecting, and the identification of endophytic actinomycetes from medicinal plant *Vochysia divergens*. The work was divided into three chapters and includes bioprospecting and avaliation of biological activity of endophytic isolates - mainly belonging to the genus *Microbispora* - followed by identification of compounds produced by the most promising strain and subsequent phylogenetic study of isolates belonging to *Microbispora* genus. The first chapter, entitled "**Antitumor, antioxidant and antibacterial activities of secondary metabolites extracted by endophytic actinomycetes isolated from *Vochysia divergens***" is about the isolation and biodiversity of actinomycetes from the medicinal plant *Vochysia divergens*, and the ability of six isolates to produce compounds with antibacterial, antioxidant and antitumor activities. The second chapter, "***Microbispora* sp. LGMB259 Endophytic actinomycete Isolated from *Vochysia divergens* (Pantanal, Brazil) Producing β-Carbolines and Indoles with Biological Activity**" aimed to isolate and identify compounds produced by the strain *Microbispora* sp. LGMB259. We identified seven compounds produced by this strain - four β-carbolines and three indoles- wherein the compound 1-vinyl-*β*-carboline-3-carboxylic acid was responsible for the biological activities of this strain, which showed antibacterial, antifungal and cytotoxic activities. The last chapter, entitled "**Multilocus Sequence Analysis of the Genus *Microbispora***" was based on multigene analysis using the 16S rRNA, 23S rRNA, *gyr*B and *rpo*B for phylogenetic studies in the *Microbispora* genus. The analyzes suggest that the species *M. amethystogenes, M. chromogenes, M. karnatakensis, M. parva, M. aerata, M. thermodiastatica* and *M. thermorosea* are distinct from *M. rosea*; different by proposed for Miyadoh et al. (1990). However *M. aerata* and *M. thermodiastatica* probably are the same species, as well *M. indica* and *M. rosea*. Endophytic isolates belongs to the clusters *Microbispora* sp. 1, *Microbispora* sp. 2 and *Microbispora* sp. 3 are different from the *Microbispora* species previous described and futures studies are required to describe these species. It is also proposed the concatenated analyses of *gyr*B-*rpo*B genes as a useful alternative to DNA–DNA hybridization for the identification and phylogenetic analysis in the *Microbispora* genus, and values less than 98,0% to characterizate and determine relationship at species level.

Key-words: *Vochysia divergens* – *Microbispora* Genus – secondary metabolites – β-carbolines – phylogenetic reclassification

SUMÁRIO

I. Justificativa

O gênero *Microbispora* foi proposto para actinomicetos que formam hifas aéreas e esporos em pares longitudinais. Um grande número de linhagens de *Microbispora* foi isolado neste estudo sobre a biodiversidade de actinomicetos endofíticos isolados da planta *Vochysia divergens*. Para a caracterização destas linhagens foi proposto primeiramente a utilização do gene 16S rRNA, porém ao realizar análises filogenéticas observou-se que as mesmas não apresentavam suporte para classificação em nível de espécie. A importância da correta classificação em nível de espécies é baseada em razões ecológicas e para fins industriais. Actinomicetos são a fonte principal de metabólitos secundários utilizados pela indústria farmacêutica. *Streptomyces* e actinomicetos isolados do solo foram severamente explorados no passado e auxiliaram na descoberta de muitos compostos, porém, devido ao amplo número de pesquisas envolvendo estes microrganismos, a probabilidade da descoberta de novos compostos a partir de linhagens isoladas do solo torna-se cada vez menos provável. Esses dados levaram a busca e outras fontes naturais para o isolamento de actinomicetos ser foco de inúmeras pesquisas. Com base nestes dados, objetivou-se esclarecer a filogenia do gênero *Microbispora* com base em análise multigênica, bem como, a bioprospecção, isolamento e identificação química de metabólitos secundários produzidos por linhagens endofíticas isoladas da planta *Vochysia divergens*.

II. Objetivos

- Bioprospectar metabólitos secundários produzidos por linhagens de *Microbispora* isoladas da planta *Vochysia divergens*;
- Identificar secundários de interesse biológico;
- Esclarecer a filogenia do gênero *Microbispora* por meio de sequenciamento multigênico e caracteres morfológicos, utilizando-se linhagens tipo das espécies;
- Identificar as espécies de *Microbispora* presentes na coleção de culturas do LabGeM/UFPR.

III. Metas

- Realizar o levantamento inicial de isolados promissores para a produção de compostos antimicrobianos e antitumorais;
- Isolar e identificar compostos químicos de interesse biológico;
- Realizar o sequenciamento dos genes 16S rRNA, *gyr*B, *rpo*B e 23S rRNA para as linhagens tipo pertencentes ao gênero *Microbispora* e isolados endofíticos presentes na coleção do Laboratório de Genética de Microrganismos (UFPR);
- Redefinir as espécies de *Microbispora* através de análise filogenética multilocos;
- Identificar gene/genes para utilização na classificação em nível de espécie, com suporte filogenético;
- Disponibilizar as sequências e os alinhamentos gerados em bancos de dados para consulta pública;
- Publicação dos resultados.

IV. Introdução

A necessidade de novos compostos para a indústria farmacêutica e agronômica tem levado a bioprospecção de microrganismos isolados de ambientes inexplorados. A resistência a medicamentos antimicrobianos ainda hoje, permanece como um dos principais problemas na área da saúde moderna, com o impacto sobre as opções de tratamento, mortalidade e controle da infecção, além de questões econômicas (UEKOTTER, 2011; PEREZ e VILLEGAS, 2015; GIRERD-GENESAY et al. 2015). A linha de pesquisa "Bioprospecção de microrganismos endofíticos de plantas medicinais" do Laboratório de Genética de Microrganismos (LabGeM) gerou um grande número de isolados endofíticos de uma grande variedade de plantas. Esses microrganismos vêm sendo testados quanto à atividade antimicrobiana frente a fitopatógenos e patógenos de interesse clínico, e quanto às atividades antitumoral e antioxidante. Dentre estes isolados se encontram as linhagens de *Microbispora* isoladas no Pantanal Sul-mato-grossense (SAVI, 2011; GLIENKE et al. 2012, SAVI et al. 2014; SAVI et al. 2015). O gênero *Microbispora* é um importante grupo de actinomicetos com potencial biotecnológico pela produção de importantes metabólitos secundários - entre os quais se incluem antibacterianos (VASILE et al. 2012; INDANANDA et al. 2013), antifúngicos (PATEL et al. 1998) e antitumorais (IVANOVA et al., 2012) – por esta razão objetivou-se a bioprospecção e identificação de metabólitos secundários produzidos por linhagens de *Microbispora* isoladas da planta medicinal *Vochysia divergens* (Pantanal, Brasil), que fazem parte da Coleção de Culturas do LabGeM.

A descrição atual para uma nova espécie de *Microbispora* é realizada através, principalmente de características morfológicas e bioquímicas, embora com pouco suporte filogenético em análises utilizando o gene 16S rRNA (NAKAJIMA et al. 1999; BOONDAENG et al. 2009; SAVI et al. 2014).

Uma das principais limitações na classificação de espécies com base em apenas caracteres morfológicos é a variação apresentada pelos indivíduos. Esta pode não representar espécies diferentes, e sim uma variabilidade fenotípica intraespecífica. E da mesma forma, indivíduos com características morfológicas semelhantes podem pertencer a espécies diferentes, especialmente quando traços fenotípicos similares estão presentes em vários grupos distintos (VINNERE, 2004). Desde 1980, com o advento das técnicas de biologia molecular, estas vem sendo utilizadas na estrutura taxonômica do gênero *Microbispora*, incluindo análises de sequências do gene 16S rRNA e hibridização de DNA (MIYADOH et al. 1990; WANG et al. 1996; ZHANG et al. 1998). No entanto, devido às lacunas em cada método, por exemplo, resolução insuficiente, extenso tempo de trabalho, altos níveis de erros no desenvolvimento e baixa taxa de reprodutibilidade, estas análises levam a muitas limitações para uso rotineiro. Além deste contexto, pode-se considerar que, as facilidades disponíveis atualmente para sequenciamento de DNA, a falta de treinamento em filogenia molecular e a extensa e confusa variabilidade morfológica de isolados de *Microbispora*, levaram ao depósito de grande número de sequências em banco de dados como o Genbank as quais, nem sempre foram corretas sob o ponto de vista de identificação de espécie. Este acúmulo de sequências erroneamente depositadas nos bancos de dados tem levado à criação de grande confusão taxonômica dentro desse grupo.

Por isso, abordagens multigênicas têm sido utilizadas como critério mais preciso para a correta classificação e em análises filogenéticas em um amplo grupo de bactérias e actinomicetos. Isto ocorre devido a sua reprodutibilidade e portabilidade entre laboratórios, e eficiência nas análises inter- e intra-específica (DEVULDER et al. 2005; LABEDA et al. 2014; TAMBONG et al. 2014; CHEN et al. 2015). Com base nos dados apresentados, no presente trabalho é proposto uma análise multilocus utilizando os genes 16S rRNA, 23S rRNA, *gyr*B e *rpo*B para esclarecer a estrutura taxonômica dentro das espécies de

Microbispora. A análise foi realizada utilizando sequências de linhagens referência pertencentes ao gênero *Microbispora*, bem como isolados endofíticos da planta *V. divergens*. Este estudo ofereceu a primeira análise multilocus para o estudo taxonômico de *Microbispora*, o que irá facilitar a compreensão da filogenia e a evolução deste gênero, para auxílio na discriminação de espécies, a descoberta e descrição de novas espécies por razões ecológicas e para fins industriais.

V. Revisão da literatura

Vochysia divergens e o Pantanal Sul Mato-grossense

O Pantanal é uma planície de inundação periódica, pertence à Bacia Hidrográfica do Rio Paraguai com uma área estimada em 361.666 km^2, que está localizado no centro da América do Sul, ocupando parte do território brasileiro e uma pequena parte do território paraguaio e boliviano, com uma área total de 147.574 km^2. O rio Paraguai atravessa a região de norte a sul e é responsável pela rede de drenagem formada pelos rios Cuiabá, São Lourenço, Itiquira, Correntes, Taquari, Negro, Aquidauana e seus afluentes (SILVA et al. 2000; SOARES et al. 2006). Devido a ampla diversidade de habitats o Pantanal foi reconhecido como um Patrimonio Natural da Humanidade na Constituição do Brasileira de 1988 e como uma região de inundação de Importância Internacional pela Convenção de Ramsar. Embora ainda considerada uma zona úmida relativamente intocada (JUNK et al. 2006), o Pantanal está atualmente sob ameaça, devido a perda de habitat e degradação da diversidade causada principalmente por pressões agrícolas que ocorrem tanto nas áreas que não sofrem inundação e, cada vez mais, dentro da própria planície de inundação (EVANS et al. 2014).

Cunha et al. (2006) em um estudo sobre a distribuição de espécies vegetais em áreas do Pantanal verificou que há influência do pulso de inundação – que ocorre de janeiro a julho – sobre a distribuição de espécies nas áreas alagáveis. Este fator pode atuar como estressor para as comunidades de plantas, bem como promotor da diversidade de habitats e espécies. Entre as espécies vegetais que respondem muito bem ao pulso de inundação do Pantanal está a planta *Vochysia divergens* popularmente conhecida como cambará. De acordo com Pott et al. (2011) esta planta é uma espécie amazônica considerada invasora nas regiões de solos argilosos e que tolera muito bem a inundação.

No Pantanal Mato-Grossense podem ser observadas extensas áreas onde *V. divergens* apresenta-se como espécie monodominante, originando áreas denominadas cambarazais. Estudos fitossociológicos realizados por Silva et al. (2000), revelaram que a área de cambarazal ocupa 3,1% da área total do Pantanal Mato-Grossense. A espécie *V. divergens* possui relevância econômica para a população pantaneira, a madeira é utilizada para fabricação de canoas e construção de ranchos, produção de tábuas, compensado e celulose (CORREA, 2007).

O cambará também é empregado, de forma considerável, como planta medicinal (folha e casca), sendo um recurso terapêutico contra doenças respiratórias e problemas gastrintestinais. Com a casca do caule prepara-se um xarope com mel com atividades expectorante, contra tosse, gripe e, também, utilizado no tratamento de apendicites. A partir das folhas são preparados chás usados contra asma, gripe e distúrbios digestivos (POTT et al. 2011).

Apesar do interesse econômico e ampla utilização medicinal do Cambará pela população, existem pouquíssimos relatos sobre a composição química e atividade biológica de *V. divergens.* No que diz respeito às atividades biológicas relacionadas à espécie, foi verificado que o extrato etanólico das cascas de *V. divergens* apresenta atividade bactericida frente à *S. aureus* devido a produção de ácido betulínico e ácido sericico (Hess et al. 1995). Extratos desta planta também apresentam atividade antinociceptiva, de forma dose-dependente, em camundongos submetidos a testes nociceptivos (dor e inflamação) e contorções abdominais pela produção do compostos Ácido tormentico (BORTALANZA et al. 2002).

Microrganismos endofíticos

O Brasil detém aproximadamente 20% de toda a biodiversidade mundial de macrorganismos, a maior do Planeta, e fonte inestimável de matérias-primas nos mais variados setores. Apesar da imensa diversidade biológica, a biodiversidade de microrganismos no Brasil é ainda considerada desconhecida, sendo evidente a necessidade de estudos sobre a biologia e funcionalidade do todo para manter um dinâmico ecossistema (PYLRO et al. 2014).

O estabelecimento de plantas em seus respectivos habitats envolve a sua capacidade em interagir com diferentes espécies de seres vivos. Dentre estas associações, destacam-se as mutualísticas com microrganismos como fungos micorrízicos e bactérias fixadoras de nitrogênio. Além destes, outros microrganismos, chamados de endofíticos têm recebido especial atenção devido à sua importância em relação a diferentes espécies vegetais (REINHOLD-HUREK e HUREK, 2011; CHADNA et al. 2015).

Microrganismos endofíticos foram descritos inicialmente por Bary em 1866, mas, somente há poucas décadas, foi demonstrado que o interior de raízes, folhas e sementes de plantas poderiam servir como reservatório para abrigar estes microrganismos (SOUZA, 2004). Pela ausência de conhecimento sobre o papel biológico destes microrganismos, alguns termos foram utilizados para defini-los. Sturdy e Cole (1974) os denominaram como microrganismos endógenos, enquanto Gardener et al. (1982) os consideraram como bactérias resistentes ao xilema. Segundo Kloepper e Beuchamp (1992), o termo mais adequado é “endofítico”, que pode ser definido como “microrganismos que ocorrem no interior dos tecidos de plantas e aparentemente não causam sintomas de doença ao hospedeiro”. Hallmann et al. (1997) sugeriram uma definição mais ampla, considerando microrganismos endofíticos aqueles isolados de tecidos vegetais desinfectados que não causam aparentemente danos a planta.

As interações endófito/planta, ainda não são muito bem compreendidas, mas podem ser simbióticas, neutras ou antagônicas (neste caso, estudadas pela fitopatologia). Nas interações simbióticas os microrganismos produzem ou induzem a produção de metabólitos primários e secundários que podem conferir diversas vantagens à planta tais como a diminuição da herbivoria e do ataque de insetos, o aumento da tolerância a estresses abióticos e o controle de outros microrganismos (CARVALHO et al. 2014; NAIR e PADMAVATHY, 2014; HAN et al. 2015).

Os microrganismos endófitos isolados de tecidos vegetais de partes aéreas, como folhas e pecíolos, apenas recentemente têm despertado o interesse da comunidade científica, especialmente por seu potencial na produção de metabólitos de interesse farmacêutico e agronômico. Podem ser utilizados como vetores para introdução de genes de interesse nas plantas (MURRAY et al., 1992), como agentes inibidores de pragas e patógenos (DI GIALLONARDO e HOLMES, 2015; GRUBISHA e COTTY, 2015) e como fontes de metabólitos primários (STAMFORD et al., 1998) e secundários de interesse como o taxol, poderoso anticancerígeno (STROBEL et al. 1999; WANG et al. 2015), a criptocandina, lipopeptídeo antimicótico (STROBEL et al., 1999) e diversos outros antibióticos.

Bioprospecção

A bioprospecção é a exploração e investigação de plantas, animais e microrganismos a fim de identificar princípios ativos úteis principalmente para a indústria farmacêutica, alimentícia e agronômica (TRIGUEIRO, 2002). A exploração de microrganismos com fim econômico e terapêutico teve início após Pasteur descobrir que o processo de fermentação era oriundo do crescimento microbiano, e sequentemente a descoberta da Penicilina por Fleming a partir da cultura do fungo *Penicillium notatum*, o que levou a valorização da busca e descoberta de novos compostos de interesse biológico (STROBEL e DAISY, 2003). Os

produtos naturais representam 60% dos compostos classificados como “new chemical entities” (NCEs) ativos contra o câncer e 75% dos ativos contra doenças infecciosas obtidas entre 1981 e 2002 (DEMAIN, 2014).

O Brasil e a Espanha destacam-se entre os países ibero-americanos pela produção do conhecimento científico em bioprospecção de sua biodiversidade (LIMA e VELHO, 2008). Apesar da ampla biodiversidade brasileira e o grande número de pesquisas sobre biodiversidade e potencial biotecnológico, não se faz presente hoje no mercado farmacêutico medicamentos oriundos da exploração da biodiversidade em nosso país. Marinho et al. (2008) ressaltam que uma maior articulação entre a pesquisa cientifica em Universidades e a iniciativa privada valorizaria a imensa biodiversidade brasileira e estimularia a indústria nacional. Um exemplo dessa alternativa são os incentivos em pesquisa e desenvolvimento nessa área disponibilizada pelo governo dos Estados Unidos, onde foram criadas leis de transferência de tecnologia pública para o setor privado e 90% das empresas executam atividades em cooperação com universidades.

Metabólitos secundários

Actinomicetos são responsáveis pela produção de uma gama diversificada de metabólitos secundários com propriedades medicinais, incluindo, antibióticos, antitumorais, agentes imunossupressores e hormônios de crescimento (STROBEL et al. 2004; INDANANDA et al. 2013; ENCHEVA-MALINOVA et al. 2015) e desempenham um papel importante na indústria farmacêutica. Os metabólitos secundários com atividade biológica produzidos pelo gênero *Streptomyces* são bastante conhecidos e já forneceram ao mercado farmacêutico várias opções terapêuticas. Novos agentes antimicrobianos são extremamente necessários para combater o número crescente de linhagens resistentes, sendo que produtos naturais continuam sendo a fonte mais propícia de antibióticos. A probabilidade da

descoberta de um novo composto tendo uma estrutura química nova pode ser aumentada com o isolamento de espécies raras, e para isso sugere-se a triagem em ambientes inexplorados. Actinomicetos raros são geralmente considerados como linhagens que apresentam frequência de isolamento menor do que linhagens de *Streptomyces* e, portanto, podem ser considerados como um recurso fecundo para o isolamento de novos compostos (TIWARI et al. 2011).

De encontro a estes dados, metabólitos secundários produzidos por linhagens de *Microbispora* já forneceram numerosos compostos com atividade biológica para a indústria farmacêutica. Entre os compostos produzidos por este gênero está o Cochinmicina que possui atividade bactericida (ZINK et al. 1992). Propeptina, um antibiótico peptídico cíclico, foi isolado da linhagem *Microbispora* sp. SNA-115 (KIMURA et al. 1997). Patel et al. (1998) em uma triagem visando a descoberta de novos antifúngicos e antibióticos, isolaram um novo composto denominado Sch 31828. Um isolado proveniente de solo da Malásia e caracterizado como *Microbispora* sp. A34030 produziu um composto que recebeu o nome de Bispolides. O composto em questão foi classificado como pertencente à classe dos macrolídeos e apresentou atividade contra *Staphylococcus aureus* Meticilina Resistente (OKUJO et al. 2007). O antibiótico Microbisporicina é um potente antibiótico com atividade contra patógenos Multirresistentes incluindo *Staphylococcus aureus* Meticilina Resistente e *Enterococcus* resistente a Vancomicina e também foi extraído de uma linhagem de *Microbispora* isolada do solo (CASTIGLIONE et al. 2008; VASILE et al. 2012).

Entre as atividades referentes a este gênero encontra-se a atividade antitumoral frente a células de leucemia humana e de carcinoma de colo do útero. Esta atividade foi relatada para o composto Dicetopiperazina o qual foi extraído da cultura de *Microbispora aerata* isolada da Antártica (IVANOVA et al. 2012).

Gênero *Microbispora*

O gênero *Microbispora* foi proposto por Nonomura e Ohara em 1971 e é caracterizado por actinomicetos que formam micélio aéreo com pares longitudinais de esporos. Este gênero contém seis espécies descritas atualmente, sendo que *Microbispora rosea* é a espécie tipo.

Em 1990 Miyadoh et al. realizaram uma revisão taxonômica do gênero *Microbispora* utilizando técnicas de quimiotaxonomia e hibridização DNA-DNA. Os autores propuseram a combinação de 10 espécies de *Microbispora* em uma única espécie, *Microbispora rosea*, utilizando como critério homologia de DNA. O gênero *Microbispora* pode ser separado em três grupos de acordo com a temperatura de crescimento e hibridização de DNA-DNA. Dentre as linhagens mesófilas se encontram *M. rosea, M. amethystogenes, M. chromogenes, M. diastatica, M. indica, M. karnatakensis* e *M. parva*. Segundo Miyadoh et al. (1990) estas espécies são intimamente relacionadas (valor de homologia de DNA entre 48 a 93%, média de 64,7%). Três espécies de *Microbispora* apresentam crescimento ótimo a 55ºC, estas são *M. aerata, M. thermodiastatica* e *M. thermorosea*. As linhagens termófilas exibem homologia de DNA entre si de 59-94%, sendo a média de 72,2%. Comparando as linhagens mesófilas e termófilas elas apresentam homologia de DNA de 37-60%; média 46,0%.

Segundo Miyadoh et al. (1990) era notável que as linhagens de *Microbispora* (mesófilas e termófilas) apresentavam valores elevados de homologia (média de 56,5%) nas combinações de sondas e alvos utilizados, e sugeriram que as mesmas deveriam ser agrupadas em uma única espécie. Os autores propuseram que a diferença na temperatura de crescimento entre linhagens termófilas e mesófilas seria a base para a discriminação em nível de subespécie. Porém, no Manual Bergey de Sistemática Bacteriológica, Johnson (1984) afirma que valores de homologia de DNA na gama de 70% seriam o ponto crítico para

delinear espécies. De acordo com essa diretriz, os valores de homologia entre as espécies de *Microbispora* são relativamente baixo para que possam ser consideradas como sinônimos.

No ano de 1993 Ochi et al. trabalharam com classificação do gênero *Microbispora* utilizando como marcador a mobilidade eletroforética da proteína AT-L30. Os autores observaram que na primeira dimensão de eletroforese as espécies de *Microbispora* apresentaram padrão de migração diferente, entretanto nas configurações bidimensionais a migração mostrou-se semelhante para as dez espécies. Assim, para uma melhor resolução os autores realizaram o sequenciamento de aminoácidos da proteína AT-L30. As sequências apresentaram alta homologia para as cinco espécies de *Microbispora* analisadas, porém também apresentaram semelhança com sequências da mesma proteína em *E. coli*. Os autores citaram que, os dados do trabalho lançam dúvidas sobre a classificação proposta por Miyadoh et al. (1990), e discutem que apesar das espécies estarem estreitamente relacionadas, não havia dados suficiente para suportar a classificação como uma única espécie.

Wang et al. (1996) realizaram o primeiro estudo filogenético utilizando o gene 16S rRNA para o gênero *Microbispora*. Os autores utilizaram 7 das 10 espécies presentes no trabalho de Miyadoh et al. (1990). As linhagens apresentaram alto valor de homologia – 94,9% - em sequências do gene 16S rRNA, e sugeriram que as mesmas poderiam ser uma única espécie. Porém, no ano de 1994 Stacketbrandt e Goebel estudaram a correlação entre sequências de 16S rRNA e resultados de análises de reassociação DNA-DNA visando determinar o limiar para definição de espécies bacterianas. Estes autores concluíram que microrganismos que exibiram valores menores que 97% de similaridade em sequências de 16S rRNA e valores de reassociação de DNA-DNA menores que 60% pertenceriam a diferentes espécies. Miyadoh et al. (1990) combinaram as 10 espécies de *Microbispora* em uma única espécie (*Microbispora rosea*), com base em um valor de parentesco de 56%, e

Wang et al. (1996) observaram valores de similaridade menores de 97%, dados estes mostram incongruências na classificação proposta por Miyadoh et al. (1990).

Nakajima et al. (1999) estudaram actinomicetos isolados do solo da Tailândia e isolaram duas linhagem pertencentes ao gênero *Microbispora*. Com base em dados morfológicos, bioquímicos e moleculares eles propuseram a espécie *Microbispora corallina.* A análise filogenética utilizando o gene 16S rRNA mostrou a formação de três clados (Figura 1). Levando em consideração os critérios utilizados pelos autores, para propor *Microbispora corallina* como uma nova espécie, torna-se improvável que as outras espécies sejam uma única espécie, *M. rosea*.

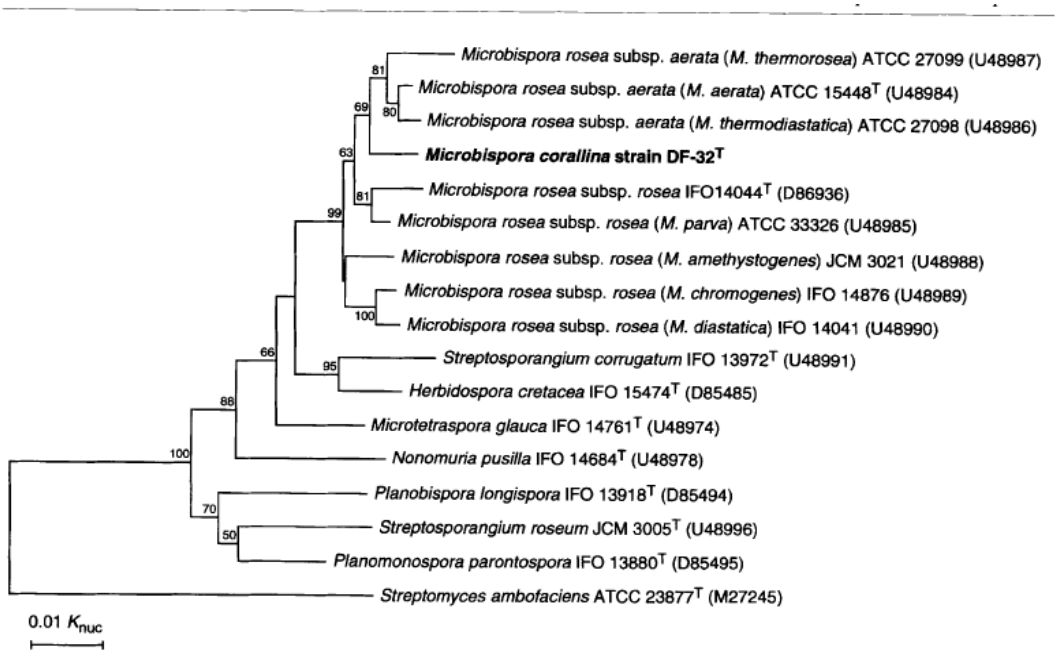

Fig. 1. 16S rDNA-based neighbour-joining tree showing the relationship between strain DF-32^T and *Microbispora* species and selected organisms belonging to the family *Streptosporangiaceae*. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates (greater than 50%). The scientific names in parentheses are the originally proposed names of the strains; the numbers in parentheses are the GenBank/EMBL/DDBJ database accession numbers.

Figura 1. Análise filogenética utilizando sequências parciais do gene 16S rDNA, baseada em Neighbour-joining utilizada para propor *Microbispora corallina* como uma nova espécie.

Fonte: Nakajima et al. (1999)

Boondaeng et al. (2009) trabalharam com actinomicetos isolados de amostras de solo do Japão e propuseram a espécie *Microbispora siamensis*. Novamente a classificação proposta por Miyadoh et al. (1990) foi utilizada, e a filogenia mostrou evidências da necessidade de reclassificação deste gênero. Na Figura 2 observa-se que a nova espécie *Microbispora siamensis* forma um clado com *M. rosea* subsp. *rosea* e *M. corallina*, também observa-se outros clados formados por *M. rosea* subsp. *rosea* e *M. rosea* subsp. *Aerata* e pela análise sugere-se que se *M. siamensis* é uma nova espécie, e diferente de *M. corallina* e *M. rosea* subsp. *rosea*, as demais linhagens caracterizadas como *M. rosea* não pertencem a mesma espécie, como proposto por Miyadoh et al. (1990) e evidenciam a falta de suporte para classificação em nível de espécie dentro desse gênero.

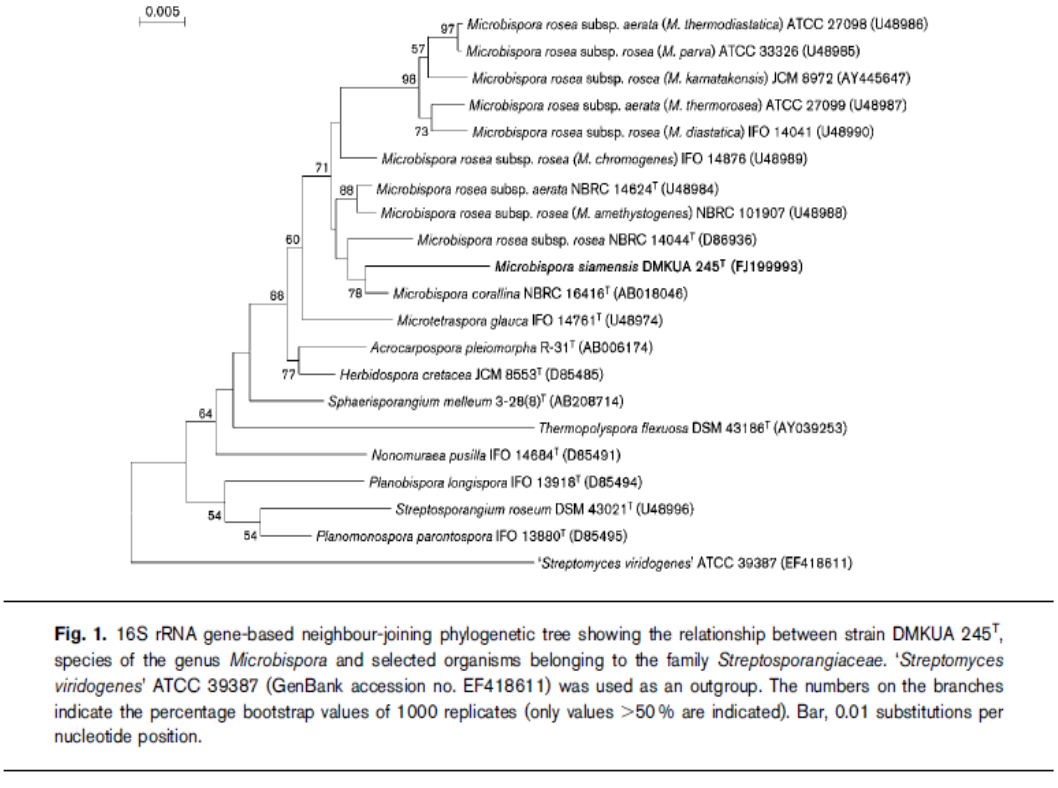

Fig. 1. 16S rRNA gene-based neighbour-joining phylogenetic tree showing the relationship between strain DMKUA 245^T, species of the genus *Microbispora* and selected organisms belonging to the family *Streptosporangiaceae*. '*Streptomyces viridogenes*' ATCC 39387 (GenBank accession no. EF418611) was used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates (only values >50 % are indicated). Bar, 0.01 substitutions per nucleotide position.

Figura 2. Análise filogenética de sequenciamento parcial do gene 16s rDNA, baseada em Neighbour-joining utilizada para propor *Microbispora siamensis* como uma nova espécie.

Fonte: Boondaeng et al. (2009)

A necessidade da reclassificação do gênero *Microbispora* é evidenciada também, pela difícil classificação em nível de espécie relatada em vários trabalhos como o de Lee et al. (2008), Hayakawa et al. (2008), Qin et al. (2009) e Savi (2011), e análises filogenéticas incongruentes mostradas anteriormente.

VI. CAPÍTULO 1 - Publicado na revista "International Journal of Pharmaceutical Chemical and Biological Sciences" (ISSN: 2249-9504)

Antitumor, antioxidant and antibacterial activities of secondary metabolites extracted by endophytic actinomycetes isolated from *Vochysia divergens*

D.C. SAVI[1]; C.W.I. HAMINIUK[2].; G.T.S. SORA; D.M. ADAMOSKI[4]; J. KENSKI [5]; S.M.B. WINNISCHOFER[5]; C. GLIENKE[1]

Affiliations: [1]Universidade Federal do Paraná, Programa de Pós-Graduação em Microbiologia, Parasitologia e Patologia, Av. Coronel Francisco Heráclito dos Santos, 210. CEP: 81531-970, Curitiba, PR, Brazil. [2]Universidade Tecnológica Federal do Paraná, Programa de Pós-Graduação em Tecnologia de Alimentos (PPGTA), BR 369 - km 0,5, CEP: 87301-006, Campo Mourão, PR, Brazil. [3]Universidade Estadual de Maringá, Programa de Pós-Graduação em Ciência de Alimentos (PPC), Av. Colombo, 5790. CEP: 87020-900, Maringá, PR, Brazil. [4]Laboratório Nacional de Biociências, LNBio-CNPEM, St. Giuseppe Máximo Scolfaro, 10.000. CEP: 13083-100, Campinas, SP, Brazil. [5]Universidade Federal do Paraná, Programa de Pós-Graduação em Bioquímica, Av. Coronel Francisco Heráclito dos Santos, 210. CEP: 81531-970, Curitiba, PR, Brazil.

ABSTRACT

Endophytic actinomycetes encompass bacterial groups that are well known for the production of a diverse range of secondary metabolites, including various antibiotics, antitumor, immunosuppressive agents, plant growth hormones, and have capacity of survive inside of plants tissues. *Vochysia divergens* is a Brazilian medicinal plant common isolated in the Pantanal region, and was focus of many researches, but the community endophytic remains unknown. Therefore, the goals of the present work were to carry out an initial assessment of antimicrobial, antitumor and antioxidant activities of crude extract produced by endophytic actinomycetes isolated from *Vochysia divergens*. Using 16S sequences, 10 isolates were classified as *Microbispora* sp. and two isolates were classified as *Streptomyces sampsonii*. The other two isolates were identified as *Micromonospora* sp. and are apparently undescribed species. The isolates were able to produce secondary metabolites with antioxidant activity, antitumor activity against of Glioblastoma cell and antimicrobial activity against bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, Methicillin-Resistant *Staphylococcus aureus* and the yeast *Candida albicans*. Taking into consideration the lack of effective medicaments for the treatment of Glioblastoma multiforme, and the increasing number of bacterial strains expressing resistance, the basic research using microorganisms from unexplored environmental can be an alternative to discover new secondary metabolites to treat these diseases.

KEYWORDS: Endophytic actinomycetes, Pantanal, Biological activity

INTRODUCTION

Endophytic actinomycetes are bacteria that reside in the internal tissue of plants via symbiotic, parasitic, or mutualistic without causing immediately negative effects[1]. Actinomycetes are well known for the decomposition of organic matter and for produce a diverse range of secondary metabolites, including antibiotics, antitumor, immunosuppressive agents and plant growth hormones[2,3]. The search for new natural products has been conducted extensively using soil actinomycetes, and this might have reduced the chance of finding new biologically active molecules from them. Thus, new microbial habitats need to be examined, in the search for new bioactive compounds[4].

We are particular interesting in microorganisms isolated from medicinal plants located in the Pantanal region (Brazil). The Pantanal is a periodic floodplain with area of approximately 138,183 km^2, belonging to the Paraguay River Basin[5]. Due to the dynamic character, few plants are able to tolerate long periods of flooding, that begins in November and in adjacent areas can last until mid-June. Among the plant species that have tolerance to high levels of flooding is Cambará - *Vochysia divergens*[6]. *V. divergens* is a medicinal plant, using to treat infection caused by *Staphylococcus aureus*, and respiratory problems[7].

Considering the associated limitations with the productivity and vulnerability of plant species as new metabolites sources, microorganisms serve as the ultimate, readily renewable, reproducible, and inexhaustible source of new structures bearing pharmaceutical potential[8,9]. Therefore, the goal of the present work was to carry out an initial assessment of antimicrobial, antitumor and antioxidant activities of endophytic actinomycetes obtained from *Vochysia divergens*.

MATERIALS AND METHODS

Isolation of endophytic actinomycetes

The *V. divergens* leaves were collected from 10 specimens located in two Pantanal regions, Nhecolândia (S18º10.07', W57º23.03') and Amolar (S20º10.10', W53º23.05') in Brazil. To the endophytic isolation, the preference was given to leaves with no marks, scratches or wounds. To eliminate epiphytic microorganisms, a purification protocol of six steps was used[10]. The leaves were fragmented and inoculated in Petri dishes with medium PDA (Potato Dextrose Agar). The plates were incubated at 28 °C for 30 days, and the growth was daily verified. The living cultures were deposited in the LabGeM collection, Federal University of Paraná, Curitiba, Paraná, Brazil (http://www.labgem.ufpr.br/).

Actinomycetes identification

Genomic DNA extraction was carried out using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to manufacturer's protocol. Amplification conditions followed Lee et al.[11] using the primers 9f (5' – GAGTTTGATCCTGGCTCAG) and 1541r (5'- AAGGAGGTGATCCAGCC) to amplify the 16S rDNA gene. Amplicons were sequenced using both PCR primers with BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analyzed on an ABI3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were compared with available sequences in the Genbank database of NCBI (http://www.ncbi.nlm.nih.gov/).

The Bayesian inference of phylogeny was made using MrBayes v3.1.2[12,13]. The putative stationary phase and burn-in were determined after multiple runs and post data analysis in Tracer v1.5[14] and AWTY[15]. The final trees were assembled in Sumtrees, from DendroPy v3.9.0 package[16]. Also, Maximum Likelihood analysis was performed, as

implemented in GARLI version 2.0[17], using default parameters and 1000 bootstraps pseudoreplicates.

Biological Activity

Production of extracts

The isolates that already had biological activity in other studies[9] were selected here for complementary analysis. Crude extracts were obtained through fermentation process, in PD (Potato Dextrose) medium under agitation for 14 days (110rpm, 36 °C). After fermentation, the mycelium was separated of fermented liquid by Whatman® qualitative filter paper, Grade 4. The fermented liquid was lyophilized, weighed, and diluted in ultrapure sterilized water (10 mg/mL).

Total phenolic compounds and antioxidant activity

Phenolic compound quantification

The phenolic compound content in the extracts was estimated by a colorimetric assay according to Singleton and Rossi[18]. The Folin–Ciocalteau method was used with Gallic acid as a standard. The absorbance was then measured at 765 nm using an UV/Vis double beam spectrophotometer T-80 (PG Instruments Limited, Beijing, China). The results were expressed as Gallic acid equivalents (GAE) using a calibration curve over the range of 5–250 ppm.

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH•) assay

The free radical scavenging activity was assessed with the DPPH• method as previously described by Mensor et al.[19]. Based on the total phenolic compound values, six deferent concentrations (50, 75, 125, 250 and 500 µg/mL in water) of the extract were used to perform the DPPH• assay.

Coupled oxidation of *β*-carotene and linoleic acid

The antioxidant activity was performed according to *β*-carotene-linoleic acid coupled oxidation assay was measured using the methodology of Emmons et al.[20] with modifications proposed by Prado[21]. Oxidation of the emulsion was monitored spectrophotometrically using an UV⁄Vis double beam spectrophotometer T-80 (PG Instruments Limited) by measuring absorbance at 470 nm over a period of 120 min. The degradation over time was nonlinear. Therefore, the antioxidant activity was expressed as percent inhibition relative to the control after incubation for 120 min using the following equation:

$$AOA = 100\ X\ \left(\frac{DR_c - DR_s}{DR_C}\right)$$

AOA stands for the antioxidant activity, DR_C is the degradation rate of the control (ln(*a*⁄*b*)⁄120), DR_S is the degradation rate of the sample (ln(*a*⁄*b*)⁄120), *a* is the initial absorbance at time zero, and *b* is the absorbance at 120 min.

Antitumor Activity

The U87MG human glioblastoma cell was seeded at a density of 1×10^4 cells into 96- well plates in 200 µL of DMEM high-glucose medium supplemented with 10% of fetal bovine serum (FBS), both obtained from Cultilab (Campinas, Brazil) and 50 µg/mL gentamycin. After 24 hours of incubation at 37°C and 5% CO^2 extracts was added to each well at the 50 ıg/mL concentrations. After 24 h or 48 h of incubation 200µL of MTT in HBSS (final concentration of 0,5mg/mL) were added to each well and incubated at 37 °C for a further 3 h. After that, the formazam crystals formed were dissolved in DMSO and the absorbance of the dissolved precipitate was measured using a Tecan-Infinite M200 microplate reader, in 550nm. The cell proliferation index was calculated as the ratio of the absorbance of extracts-treated cells to that of control cells. The assay was conducted five times for each cell line[22].

Antibacterial Activity

Bioautographic TLC agar-overlay assay

The antibacterial potential of the methanolic extracts of Actinomycetes endophytic was assessed against the following test organisms: *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and methicillin-resistant *Staphylococcus aureus* (MRSA). The test organisms were grown overnight in a Mueller–Hilton broth (Merck) at 37 °C and were diluted until reaching the concentration of 10^6 cells/mL. The bioautography followed the protocol described by Rahalison et al.[23].

Statistical analysis

To perform the statistical analyzes, we used the software R 3.0.0. The normality tests followed the methodology of Shapiro-Wilk test. Once the data was classified as normal was applied to parametric analysis of variance of a factor with post-hoc Tukey's HSD.

RESULTS AND DISCUSSION

Isolation and molecular characterization

Eighteen actinomycetes isolates were isolated from 4.000 analyzed leaves fragments. 55,5 % of isolates were collected in Nhecolândia and 45,5% in Amolar. 61.1% of them were obtained from stems and 38.9% from leaf tissues. The frequency of isolation was 0.47% using leaves and stem fragmentation. Actinomycetes are the largest and most dominant, comprising nearly 50% of the total population, of soil and root inhabiting, as probably saprophytes[24]. Moreover, Actinomycetes colonization in leaves and stems is less frequent. In the present study, the genera *Streptomyces* (two isolates) and *Micromonospora* (two isolates) were isolated from different regions in Pantanal: *Streptomyces* from Nhecolândia, and

Micromonospora from Amolar. *Microbispora* isolates (14 isolates) were isolated of both regions, Nhecolândia and Amolar, probably due to their higher frequency of isolation in the leaves and stem tissues of *V. divergens* (77.78%).

The partial sequence of 16S rRNA gene revealed that the isolates Clade 2 belong to the genus *Microbispora* (Figure 1). However, the isolates formed a clade with two type strains, *Microbispora rosea* (D86936) and *Microbispora mesophila* (AF002266). The phylogenic analysis also shows that using only the 16S rRNA gene, it was not possible to assign a single species to these isolates, due of conflicting topologies and no support to classification in species level. So we assumed that the 16S rRNA analysis is more appropriate for discrimination on the genus level. The isolates LGMB260 and LGMB261 were identified as *Micromonospora* sp. however, the obtained sequences were not similar to any of the available sequences from the type species of the GenBank data base (Figure 2), and probably is new specie. The isolates LGMB262 and LGMB263 were identified as *Streptomyces sampsonii* with a high degree of similarity (Figure 3). This species has been described producing an antibiotic of the polyenes group in the methanolic extract, with antifungal activity[25].

Antioxidant activity

In this study, the concentration of total phenolic ranged from 0.000337 mg/g (gallic acid equivalent) of endophytic extract LGMB262 (*Streptomyces sampsonii*), by 228.6364 mg/g of the extract from isolated LGMB259 (*Microbispora* sp.) (data not shown).

In vitro antioxidant activity of the isolates was determined by DPPH free radical scavenging ability. This technique had already been proven as a key method for detection and evaluation of antioxidant property of any molecules. The crude extracts from *Microbispora* sp. LGMB255, LGMB258 and LGMB259 had a noticeable DPPH free radical activity with

EC50 of 163,90 µg/mL, 179,04 µg/mL and 153,24 µg/mL respectively (Figure 4). It was also observed that the DPPH scavenging activity was increased in a dose-dependent manner. For comparison, in a study performed by Mahapatra and Banerjee[26], of the crude extract from *Fusarium solani* showed a protective activity of 50% (EC50) in concentration of 578.541 µg/mL and which is comparable with standard antioxidant Vit-C 433.099 µg/mL. The extracts evaluated in our present study had better protective action even in lower concentration EC50 of 153.24 µg/mL (Figure 4).

The isolate LGMB255 (*Microbispora* sp.) showed strong inhibition of lipid peroxidation with EC50 of 181.68 µg/mL. The inhibition of *β*-carotene bleaching by the isolate LGMB255 (*Microbispora* sp.) was higher than produced by fungi and bacteria[27], and equivalent to the results reported by Chen et al.[28], who studied the antioxidant activity of secondary metabolites from a strain of endophytic *Aspergillus* sp. Therefore, the extracts of endophytic actinomycetes could become an alternative over synthetic antioxidants, as butylated hydroxytoluene (BHT) and butylated hydroxanisole (BHA). Compounds reported as carcinogenic and hepatotoxic[26].

Antitumor activity

The biological effects of the metabolites was also tested against tumor cell lines, to check if beyond a protective action by production of antioxidants compounds the extracts also have activity against tumors cells. Crude extracts from *Microbispora* sp. LGMB259, LGMB250, LGMB255 and LGMB256 showed antitumor activity against Glioblastoma multiforme cell higher than 98%. The extracts of the strains LGMB258 (*Microbispora* sp.) and LGMB262 (*Streptomyces sampsonii*) showed activity of 59% and 70% percent respectively (Figure 5). Glioblastoma multiforme (GBM) represents the most aggressive tumor among high grade gliomas (HGG), with a poor prognosis of about 14-15 months[29], and has been shown to be resistant to standard therapy, either because of distinct biophysical and

genetic properties, or possibly due to migration outside of the treatment field[30]. Seznec et al.[31] in study with mithramycin compound isolated of Streptomyces strain, showed the activity in less concentration from glioblastoma cell line, by inactivation of enzyme Sp1. Thus, it is necessary to consider multimodal strategies that maximize the potency of available treatments through complementary and synergistic effects.

Antibacterial activity

Among the diseases that cause highs costs in public health, the bacterial infections have a great relevance, and this problem is increased by the developing of resistance[32]. For example, studies reporting that MRSA caused 250,000-300,000 hospital acquired infections[33]. The genus *Streptomyces* was showed many compounds for pharmaceutics industry, for example Vancomycin that was antibiotic chose for infections with bacteria multiresistance drugs[34]. Therefore, the search for new compounds can focus on the isolation of rare actinomycetes, among which, the genus *Microbiospora*[35]. Strains *Microbispora* sp. (LGMB250 and LGMB259) and *Streptomyces sampsonii* (LGMB262) showed activity against *Candida albicans* (Figure 6). Crude extract from LGMB255 (*Microbispora* sp.) also showed activity against to *S. aureus* and *E. coli* and metabolites from LGMB259 (*Microbispora* sp.) had antimicrobial activity against all tested microorganisms, including the bacteria Methicillin-resistant *S. aureus* (Table 1).

Our results show that *Microbispora* was the predominant genus of endophytic actinomycetes isolated from *V. divergens*. *Micromonospora* isolates probably belong to a new species, and a multigene analyzes is necessary to their identification. The actinomycetes isolated in this study showed a promising biological activity, a notable antioxidant and antitumor activities, and the extract from *Microbispora* sp. LGMB259 had activity against Methicillin resistant *S. aureus* which is leading cause of nosocomial infections worldwide.

These results evidence that isolation of microorganisms from unexplored environments can be one alternative to isolation of metabolites with wide biological activity.

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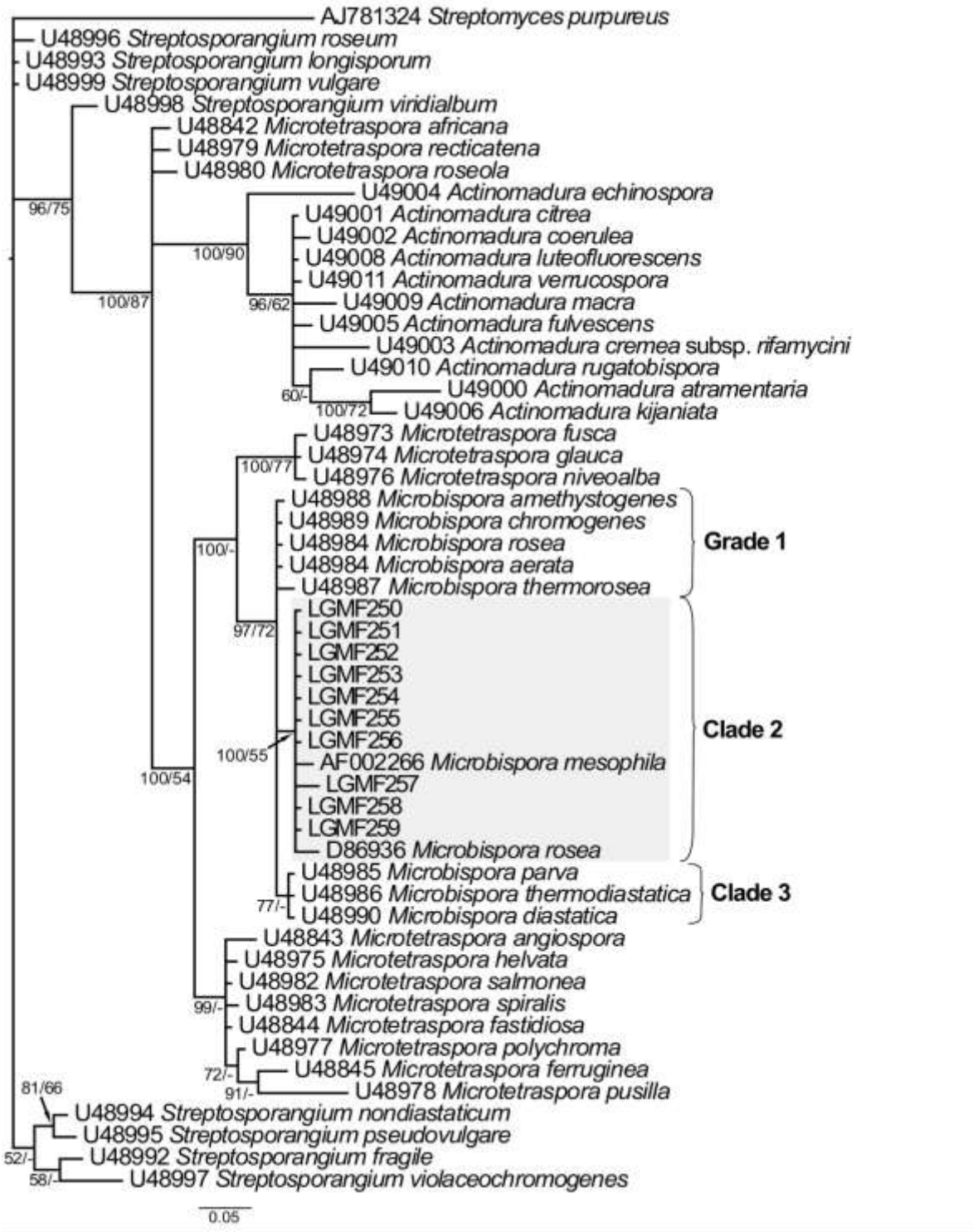

Figure 1 – Bayesian Phylogenetic tree for *Microbispora* genus using 16S rDNA gene. Posterior probabilities are shown on nodes, together with maximum likelihood bootstrap support, if it exists. Only clades with more than 50% of posterior probabilities are shown. The tree was rooted with *Streptomyces purpureus* (AJ781324). The gray shade indicates the endophytic isolates analyzed in this study.

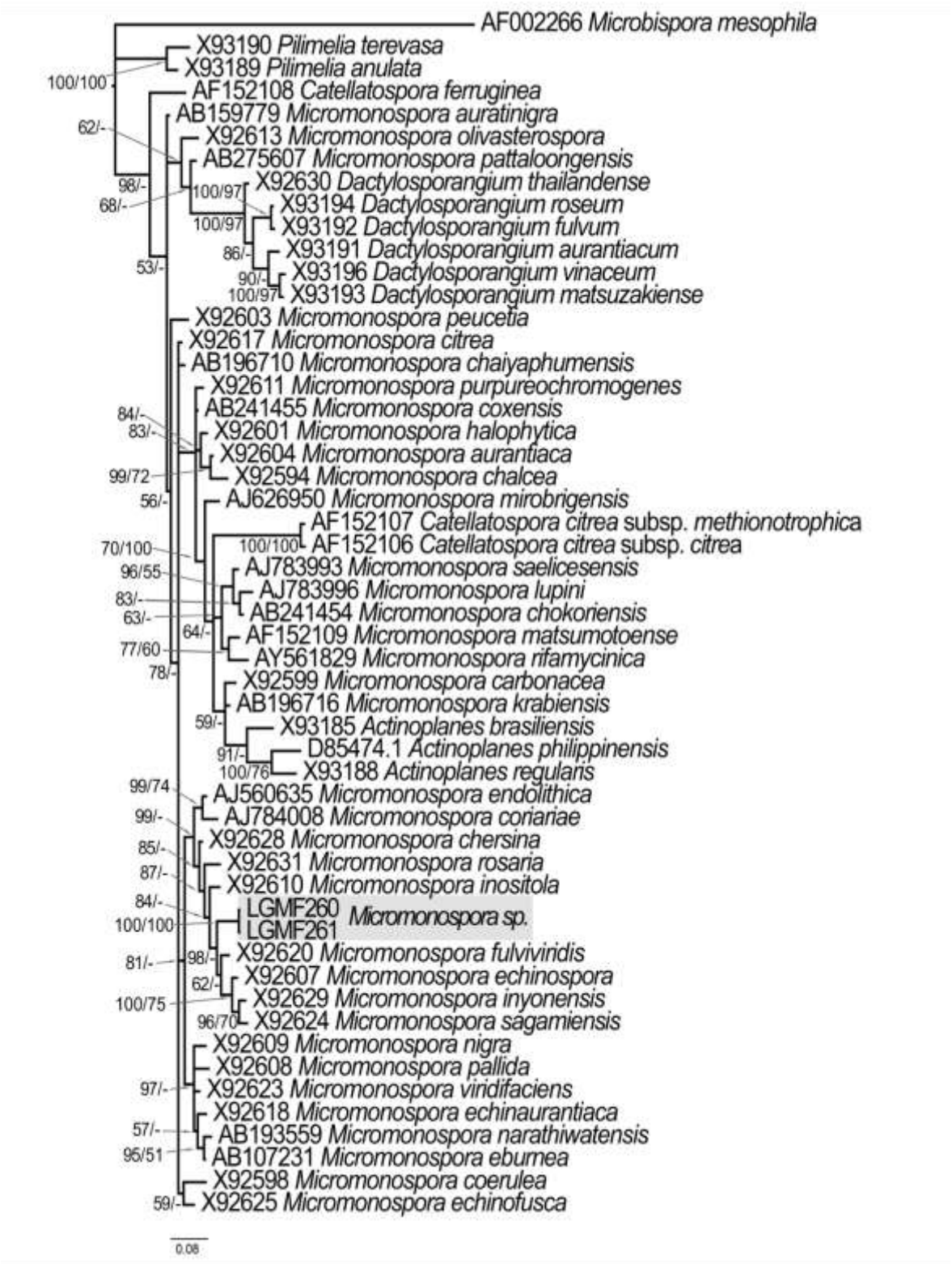

Figure 2 – Bayesian Phylogenetic tree for *Micromonospora* genus using 16S rDNA gene. Posterior probabilities are shown on nodes, together with maximum likelihood bootstrap support, if it exists. Only clades with more than 50% of posterior probabilities are shown. The tree was rooted with *Microbispora mesophila* (AF002266). The gray shade indicates the endophytic isolates analyzed in this study.

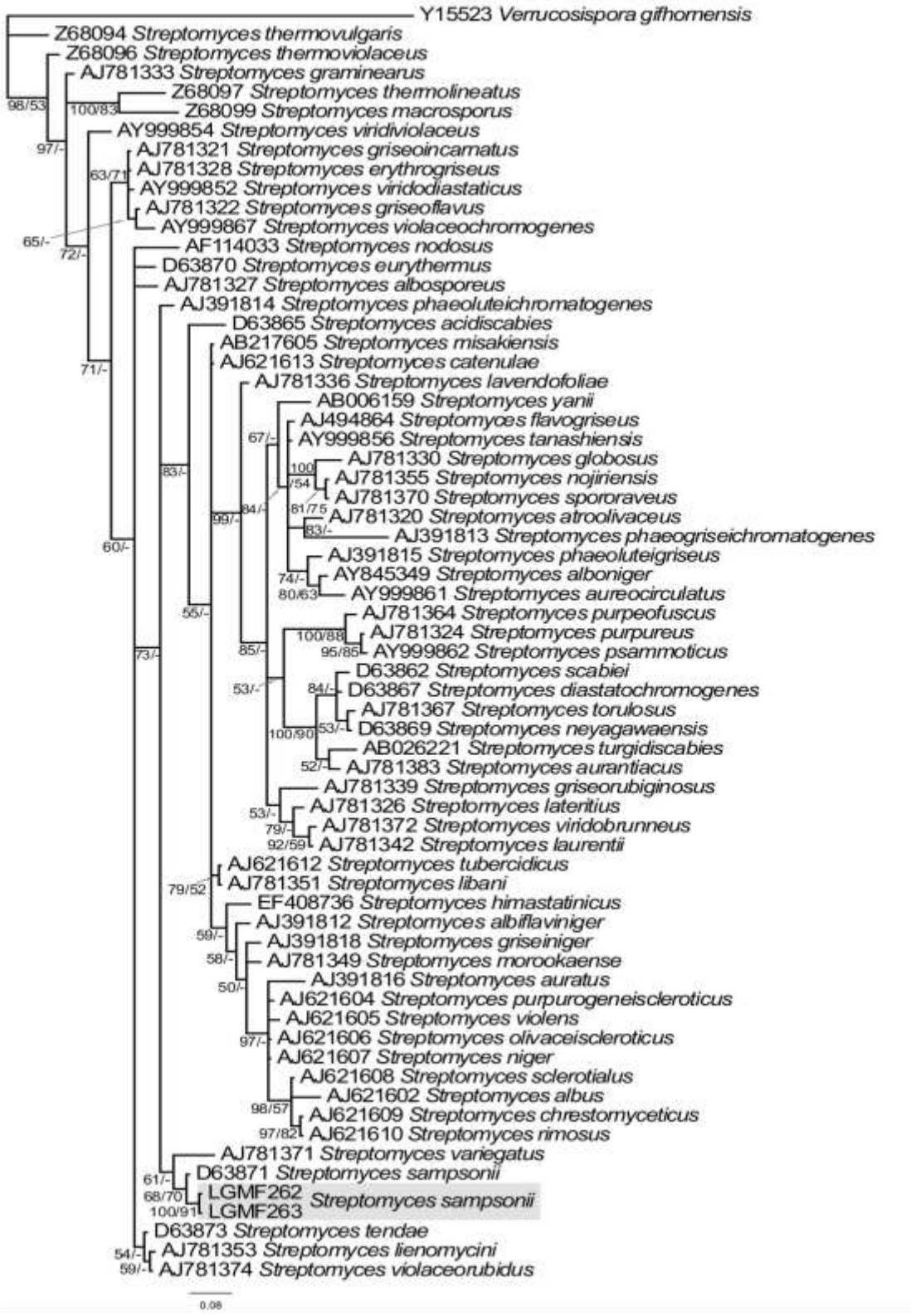

Figure 3 – Bayesian Phylogenetic tree for *Streptomyces* genus using 16S rDNA gene. Posterior probabilities are shown on nodes, together with maximum likelihood bootstrap support, if it exists. Only clades with more than 50% of posterior probabilities are shown. The tree was rooted with *Verrucosspora gifhomesis* (Y1552302266). The gray shade indicates the endophytic isolates analyzed in this study.

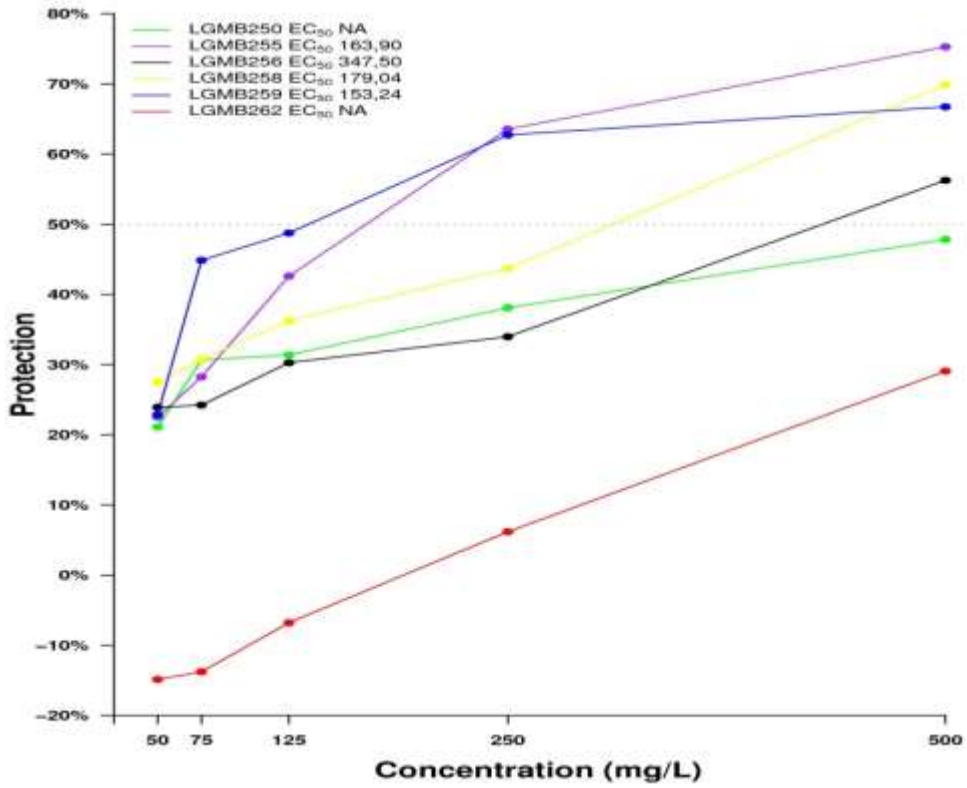

Figure 4 – Antioxidant activity evaluation of crude extracts of the endophytic actinomycetes in different concentration (Y axis represents the percentage of protection, X axis represents the extract concentrations); NA: not achieved 50%.

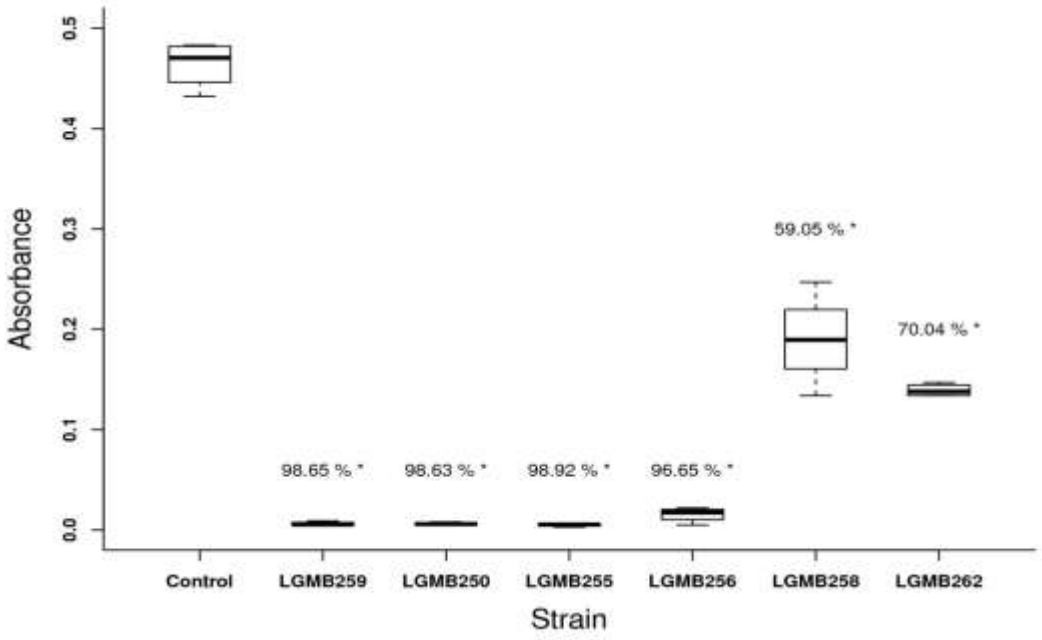

Figure 5 – Evaluation of the antitumor activity of crude extracts of actinomycetes in the concentration of 50 ıg/mL (Y axis represents the absorbance of the growth of tumor cells, de X axis represents the strain codes, * represents inhibition of growth).

Table 1. Evaluation of antibacterial activity of crude the extracts of endophytic actinomycetes by the Bioautographic TLC agar-overlay assay

	Antibacterial activity									
	Staphylococcus aureus		*Escherichia coli*		*Pseudomonas aeruginosa*		Methicillin resistant *Staphylococcus aureus*		*Candida albicans*	
Exctract	5 ul	10ul	5 ul	10ul	5 ul	10ul	5 ul	10ul	5 ul	10ul
LGMB258	0	0	0	0	0	0	0	0	0	0
LGMB256	0	0	0	0	0	0	0	0	+	+
LGMB250	0	0	0	0	0	0	0	0	0	0
LGMB262	0	0	0	0	0	0	0	0	+	+
LGMB255	+	+	+	+	0	0	0	0	0	0
LGMB259	+	+	+	+	+	+	+	+	+	+

Note: 0, No inhibition; +, inhibition zone between 4 and 5 mm in diameter.

VII. CAPÍTULO 2 – Publicado na revista "Current Microbiology" (ISSN: 1432-0991)

Microbispora sp. LGMB259 Endophytic Actinomycete Isolated from _Vochysia divergens_ (Pantanal, Brazil) Producing _β_-Carbolines and Indoles with Biological Activity

Daiani C. Savi [a,b,†], Khaled A. Shaaban[a,c,†], Nathalia Vargas[d], Larissa V. Ponomareva[a,c], Yvelise M. Possiede[e], Jon S. Thorson[a,c], Chirlei Glienke[d#] and Jürgen Rohr[a#]

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0596, U.S.A.[a]; Department of Microbiology, Parasitology and Pathology, Universidade Federal do Parana, Av. Coronel Francisco Heráclito dos Santos, 210. CEP: 81531-970, Curitiba, PR, Brazil[b]; Center for Pharmaceutical Research and Innovation (CPRI), College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0596, U.S.A.[c]; Department of Genetics, Universidade Federal do Parana, PO.BOX 19071. CEP: 81531-970, Curitiba, PR, Brazil[d]; Department of Biology, Universidade Federal do Mato Grosso do Sul, CEP: 79070-900, Campo Grande, MS, Brazil[e].

[†] Authors contributed equally to this work.

Correspondencing authors: Chirlei Glienke, +5533611562, ch.glienke@gmail.com; Jürgen Rohr, +1 859 257 7564, jrohr2@email.uky.edu

Abstract

Endophytic actinomycetes encompass bacterial groups that are well known for the production of a diverse range of secondary metabolites. *Vochysia divergens* is a medicinal plant, common in the "Pantanal" region (Brazil) and was focus of many investigations, but never regarding its community of endophytic symbionts. During a screening program, an endophytic strain isolated from the *V. divergens*, was investigated for its potential to show biological activity. The strain was characterized as *Microbispora* sp. LGMB259 by spore morphology and molecular analyze using nucleotide sequence of the 16S rRNA gene. Strain LGMB259 was cultivated in R5A medium producing metabolites with significant antibacterial activity. The strain produced 4 chemically related *β*-carbolines, and 3 Indoles. Compound 1-Vinyl-*β*-carboline-3-carboxylic acid displayed potent activity against

the Gram-positive bacterial strains *Micrococcus luteus* NRRL B-2618 and *Kocuria rosea* B-1106, and was highly active against two human cancer cell lines, namely the prostate cancer cell line PC3 and the non-small-cell lung carcinoma cell line A549, with IC_{50} values of 9.45 and 24.67 µM, respectively. 1-Vinyl-*β*-carboline-3-carboxylic acid also showed moderate activity against the yeast *Saccharomyces cerevisiae* ATCC204508, as well as the phytopathogenic fungi *Phyllosticta citricarpa* LGMB06 and *Colletotrichum gloeosporioides* FDC83.
Keywords: Pantanal; *β*-carboline; Indoles; antibiotics; anticancer agents; Endophytic *Microbispora*

Introduction

It has been well established that microorganisms are a virtually unlimited source of natural products, many of which have potential therapeutic applications. Without such discoveries "there would be a significant therapeutic deficit in several important clinical areas, such as infectious and cardiovascular disease, most solid tumors, and immune-inflammatory diseases" [24]. Therefore, there is an urgent need to search for effective new antibacterial or antifungal agents in treatment of infectious diseases at present. While terrestrial microorganisms were largely explored over the past 50 years, endophytes isolated from medicinal plants became of significant importance for the production of new compounds [4, 16]. Endophytic actinomycetes are Gram-positive bacteria reside in the internal tissues of plants via symbiotic, parasitic, or mutualistic means, without causing immediately overt negative effects for the plant [25]. The genus *Microbispora* was originally described for actinomycetes that produce characteristic paired spores on the aerial mycelium. Members of the genera *Microbispora* were isolated from various hosts, and are known for production of bioactive secondary metabolites, with antibacterial [10], antifungal [22] and antitumor [15] activities. We were particularly interested in endophytes from medicinal plants found in the Pantanal, a unique tropical wetland region of Brazil that stretches also into Bolivia and Paraguay. Due to the dynamic character of Pantanal, few trees are able to tolerate long periods of flooding, that begins in November and in adjacent areas can last until mid-June. Among the plant species that have tolerance to high levels of flooding is the *Vochysia divergens* [1]. *Vochysia divergens* is a medical plant, common in South America, and it is largely used because of its bactericide activity against *Staphylococcus aureus* and its antinociceptive activity [5].

In the present work, we describe the isolation of an endophytic actinomycete strain from *V. divergens* (Pantanal, Brazil), which generates compounds with biological activity, and the identification of this strain based on spore characteristics and phylogenetic analyze using 16S rRNA. Fermentation of this strain on R5A-medium, followed by extraction and purification yield 4 *β*-carbolines and 3 indoles (Fig. 1). Compound 1-vinyl-*β*-

carboline-3-carboxylic acid showed large biological activity, in contrast the compounds JBIR-133, kitasetaline and methyl 1-(propionic acid)-*β*-carboline-3-carboxylic acid neither revealed activities. The bioactivity tests indicate that the vinyl side chain attached at 1-position in compound **1** is essential for both the antitumor and the antibiotic activity of this natural product, and the studies presented here provide further insights into the structure activity relationships (SAR) of *β*-carbolines.

Material and Methods

Taxonomy

Strain LGMB259 was isolated by *V. divergens* leaves collected from the Pantanal, in the region of Nhecolândia (S18º10.07', W57º23.03') in Brazil. To the endophytic isolation, the preference was given to leaves with no marks, scratches or wounds. To eliminate epiphytic microorganisms, a purification protocol of six steps was used [23]. The leaves were fragmented and inoculated in Petri dishes with medium PDA (Potato Dextrose Agar). The plates were incubated at 28 °C for 30 days, and the growth was daily verified. The living cultures were deposited in the LabGeM collection, Federal University of Paraná, Curitiba, Paraná, Brazil (http://www.labgem.ufpr.br/).

To the Scanning Electron Microscope strain LGMB259 was grow up in plates ISP medium 3 [12] at 37 °C for 15 days, and was fixed in Karnovsky solution (glutaraldehyde 2.5%, paraformaldehyde 2.5% in sodium cacodylate buffer 0.05 M, CaCl2 0.001 M, pH 7.2) for 24 hours. Sample was dehydrated in ascending series of ethanol, 30, 50, 70, 90 and 100% for 10 minutes at each step, the last step 100%, repeated three times. The acrylic resin infiltration was started with a pre-infiltration of PA resin and ethanol in the ratio 1:1 for approximately 5 hours, followed by infiltration with pure resin for one night. Finally, the sample was placed at room temperature for polymerizing. The analysis of the strain was performed under light microscope 'Zeiss Axioskop 2', by acquiring photographs in digital camera.

Genomic DNA extraction was carried out using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to manufacturer's protocol. The primers 9f (5' – GAGTTTGATCCTGGCTCAG) and 1541r (5'- AAGGAGGTGATCCAGCC) were used to amplify the gene 16S rRNA [20]. The PCR product was purified using ethanol precipitation. The product of PCR was sequenced using BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analyzed on an ABI3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The sequence was compared with available sequences in the Genbank database of NCBI

(http://www.ncbi.nlm.nih.gov/), and was aligned using the CLUSTAL_W v.1.81 program [27]. Alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed using the Maximum likelihood method in the Garli version 2.0 [28]. The confidence values for branches of the phylogenetic tree were determined using bootstrap analyses based on 1000 resampling.

Fermentation, Extraction and Isolation

The *Microbispora* sp. LGMB259 was cultivated on ISP3-agar plates at 37 °C for 7 days. Chunks of agar with the fully-grown strains were used to inoculate five (250 mL) Erlenmeyer flasks, each containing 50 mL of R5A medium [11]. Individual cultures were grown at 37 °C for 3 days and subsequently used as seed cultures for the scale-up fermentation. The seed cultures were used to inoculate 80 Erlenmeyer flasks (250 mL) each containing 100 mL of R5A medium. Fermentation (8 L) was continued at 37 °C with, shaking (250 rpm) for 10 days. The obtained orange culture broth was centrifuged and filtered over celite. The biomass (mycelium) was extracted with MeOH (5 X 500 mL) and then the recovered organics were evaporated *in vacuo* at 40 °C to yield 5.4 g of crude extract. The supernatant was mixed with 5% (w/v) XAD-16 resin and stirred overnight, followed by filtration. The water fraction was extracted with EtOAC (5 X 500 mL) and then recovered organics were evaporated *in vacuo* at 40 °C to yield 170 mg of water extract. The resin was washed with water (3 X 600 mL) and then extracted with MeOH until the eluent was colorless. The MeOH was subsequently mixed with water and extract with EtOAC (5 X 500 mL), and then recovered organics were evaporated *in vacuo* at 40 °C to yield 480 mg of XAD extract. The crude extract was then subjected to a Reverse Phase C18 column chromatography (20 X 8cm, 250g) eluted with a gradient of H_2O-MeOH (100:0-0:100), followed of HPLC purification to yield the compounds **2** (11 mg) and **3** (9.8 mg). The water extract was subjected to semipreparative HPLC and resulted in compounds **5** (10.8 mg) and **6** (12.3 mg) in pure form. The XAD extract was subject to HPLC, Sephadex LH-20 (MeOH; 1 × 20 cm), and further purified by HPLC and offered compounds **1**, **3**, **4**, **7** (11 mg, 40 mg, 11 mg, 2.3 mg , 9.6 mg) (Supplementary file, Fig. S2).

For preparative scale separation, Phenomenex (Torrance, CA 90501-1430) C18 column (10 × 250 mm, 5 µm) was used on a Varian (Varian, Palo Alto, CA, USA) ProStar Model 210 equipped with a photodiode diode array detector and a gradient elution profile (**s**olvent A: H_2O, solvent B: acetonitrile; flow rate: 5.0 mL min-1; 0-2 min 85% A and 15% B, 2-23 min, 85-20% A, 23-24 min 20% A and 80 % B, 24-25 min 20-85% A, 25-26 min 85% A and 15% B). UV spectra were recorded on an Ultrospec 8000 spectrometer (GE, Pittsburgh, USA). HRESI mass spectra were recorded on AB SCIEX Triple TOF® 5600 System. HPLC-MS analyses were carried out in Waters 2695 LC module (Waters corp. Milford, MA, USA) using a Symmetry Anal C_{18} 5 µm column (4.6 × 250

mm, Waters corp. Milford, MA 01757) and a gradient elution profile (solvent A: H_2O, solvent B: acetonitrile; flow rate: 0.5 mL min-1; 0-4 min 90% A and 10% B, 4-22 min, 90-0% A, 22-27 min 0% A and 100% B, 27-29 min 0-90% A, 29-35 min 90% A and 10 % B). NMR spectra were measured on a Varian VnmrJ 500 (^{1}H, 500 MHz; ^{13}C, 125.7 MHz) spectrometer; the δ-values were referenced to the respective solvent signals. All solvents used were of ACS grade and purchased from the Pharmco-AAPER (Brookfield, CT). R_f values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). Size exclusion chromatography was performed on Sephadex LH-20 (GE Healthcare).

Antimicrobial and Antifungal Activity

The Gram-negative bacterium *Escherichia coli* DH5α (Invitrogen) and the Gram-positive bacteria *Micrococcus luteus* NRRL B-2618 and *Kocuria rosea* B-1106 were maintained in lysogeny broth (LB) liquid media and Mueller-Hinton agar. A sterile loopful of each organism was inoculated into a 7 mL culture of LB broth and incubated in a 37 °C orbital shaker at 200 rpm for 10 hours. Each test organism was streaked on a sterile Mueller-Hinton agar plate with a sterile cotton swab. Compounds **1**-**3** were dissolved in methanol and were aliquoted in 100 μg amounts per each 6 mm sterile filter disc and were allowed to dry in a laminar flow hood. The discs were placed on the plates, which were then incubated for 24 hours at 37 °C. The resulting of inhibition zone was measured in millimeters.

The fungal strains *Saccharomyces cerevisiae* ATCC 204508, *Phyllosticta citricarpa* LGMB06 and *Colletotrichum gloeosporioides* FDC83 were used in disc diffusion assays. Solutions of amphotericin B and test compounds were made in MeOH. Each sterile paper disc was loaded with 20 μL solution and was allowed to dry in the biosafety cabinet for 4 h. The dried discs were then placed on the V8 agar plate following the homogeneous distribution of fungus. MeOH was used as a negative control. The plates were then incubated at 24 ºC for 3, 7, 10 and 14 days. Inhibition zone were then measured.

Cell Viability Assay

Conversion of resazurin (7-hydroxy-10-oxido-phenoxazin-10-ium-3-one) to its fluorescent product resorufin was monitored to assess viability of human lung non-small cell carcinoma 549 and prostate adenocarcinoma PC3 cell lines. DMEM/F-12 Kaighn's modification media (Life Technologies, NY, USA) was used to grow A549 and PC3 cells (ATCC, Manassas, VA, USA) with 10% heat-inactivated fetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 μg mL^{-1}, streptomycin, 2 mM L-glutamine. Cells were seeded at a density of 3×10^3 cells per well in 96-well clear bottom culture plates (Corning, NY, USA), incubated 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2 and were subsequently exposed to known toxin (1.5 mM hydrogen peroxide, 10

μg mL^{-1} actinomycin D) and test compounds for two days. To assess cell viability, 150 μM of resazurin (Sigma, St. Louis, MO, USA) was added to each well, plates were shaken briefly for 10 seconds and incubated for another 3 hours at 37 °C to allow viable cells to convert resazurin into resorufin. The fluorescence intensity for resorufin was detected on a scanning microplate spectrofluorometer FLUOstar Omega (BMG Labtech, Cary, NC, USA) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Results

Strain Isolation and Cultivation

In our search about biodiversity and bioactive compounds, the strain LGMB259 was isolated from leaf tissues of *V. divergens*. Strain LGMB259 produced branched and non-fragmented substrate mycelia. Non-motile spores in characteristic longitudinal pairs were borne on short sporophores branching from aerial hyphae. Each spore was oval and its surface was smooth (Fig. 2). Neither sporangium-like bodies nor any other special structures were observed, characteristic of the genus *Microbispora*. Strain LGMB259 was confirmed to belong to the *Microbispora* genus by 16S rRNA analysis. The strain showed the highest level similarity with sequences deposited in the Genbank (99%); strain *Microbispora* sp. H347, *Microbispora* sp. CRCB5 and type strain *Microbispora rosea* subsp. *rosea* JCM8971. However, in the phylogenetic analysis (Fig. 3) was not possible to assign this isolate to a single species, due the low Bootstrap support.

Structure Elucidation

A large-scale fermentation of the strain in R5A-medium afforded 5.4 g of crude extract, 170 mg of water extract and 480 mg of XAD extract. Isolation and purification of the obtained extracts using various chromatographic techniques afforded compounds **1~7** in pure forms (Supplementary file, Fig. S2).

The physicochemical properties of compounds **1**~**4** are summarized in Table 1. Compound **1** was isolated as pale yellow solid (4.1 mg L^{-1}), the molecular formula of compound **1** was deduced as $C_{14}H_{10}N_2O_2$ on the basis of HR-ESI-MS (Table 1). The proton NMR spectrum of **1** in DMSO-d_6 (Table 2) displayed one broad H/D exchangeable signal at δ 12.21 of an OH or NH group. In addition, the ^{1}H NMR spectrum displayed four aromatic proton signals at δ 8.39 (d, J = 8.0 Hz), 7.67 (dd, J = 7.5, 1.0 Hz), 7.63 (ddd, J = 8.0, 7.5, 1.5 Hz), and 7.33 (ddd, J = 8.0, 8.0, 1.0 Hz), representing a di-substituted benzene along with one aromatic proton singlet at δ 8.82. Three additional olefinic proton signals were observed at δ 7.46 (dd, J = 17.0, 10.5 Hz), 6.68 (dd, J = 17.0, 1.5 Hz) and 5.77 (dd, J = 11.0, 1.5 Hz). The ^{13}C NMR/HSQC spectra (Table 2) along with the UV spectrum confirmed the structure of compound **1** to be a β-carboline bearing a vinyl-side chain. The ^{1}H-^{1}H COSY and

HMBC correlations (Fig. 4) of compound **1** finalized the structure, showing $^{3}J_{C\text{-}H}$ and $^{2}J_{C\text{-}H}$ HMBC correlations from the CH_2-2′ and CH-1′ to C-1, respectively, confirming the attachment of the vinyl group at the C-1 position (Supplementary file, Fig. S3-13).

The molecular weights of compounds **2**, **3** e **4** were determined as 284, 401 and 299 Daltons, respectively, based on the (+) and (−)-APCI-MS (Table 1). The proton NMR spectrum along with the ^{13}C NMR/HSQC spectra (Table 2) of compounds **2**, **3** and **4** showed that they contains the same *β*-carboline core as compound **1** with *Δm/z* = 46, 163 and 60 *amu* higher than **1**, respectively. Furthermore, the signals of the vinyl group characteristic for compound **1** were missing in the spectra of compounds **2**, **3** and **4**, and instead two triplet methylene signals were observed (Table 2). Thorough analyses of the 1D and 2D NMR spectra (Table 2, Fig. 4) of compounds **2**, **3** and **4**, followed by a substructure search in AntiBase [18] revealed the identity of **2**, **3** and **4** with JBIR-133, kitasetaline and methyl 1-(propionic acid)- *β*-carboline -3-carboxylic acid, respectively (Supplementary file, Fig. S14-31).

Compounds **5~7** were characterized by mass spectra and NMR data as indole-3-carbaldehyde (**5**), indole-3-carboxylic acid (**6**) and indole-3-acetic acid (**7**), according to the data in the literature [18] (Supplementary file, Fig. S32-40).

Antibacterial and Antifungal Activities

The antibacterial activity of the crude extracts and *β*-carboline compounds (**1**~**3)** were determined against the Gram-negative and Gram-positive bacteria (Table 3). 1-vinyl-*β*-carboline-3-carboxylic acid (**1**) displayed potent antibacterial activity against the Gram-positive bacterial strains *K. rosea* and *M. luteus.* Compound **1** also showed moderate antifungal activity against *C. gloeosporioides, P. citricarpa* and *S. cerevisiae* (Table 3), while the other congeners (compounds **2**~**3)** neither revealed antibacterial nor antifungal activities against the above mentioned bacterial and antifungal strains at 100 μg/disc.

Cytotoxicity Assays

The cytotoxic activities of 1-vinyl-*β*-carboline-3-carboxylic acid, JBIR-133 and kitasetaline were determined using PC3 (prostate) and A549 (non-small cell lung) human cancer cell lines (Fig. 5A and 5B). Cell viability assays showed that 1-vinyl-*β*-carboline-3-carboxylic acid was highly active against both PC3 (IC_{50} = 9.45 μM) and A549 (IC_{50} = 24.67 μM) cell lines. In contrast, JBIR-133 and kitasetaline were not active up to 100 μM.

Discussion

This research represents the very first attempt to isolate endophytic actinomycetes from *V. divergens*, which is a typical herbal medicine, which produces compounds with antibacterial activitiy like sericic acid [14] and anti-

allodynic properties like tormentic acid [5]. This strain LGMB259 showed morphology characteristic of *Microbispora* genus. Highs values (99%) of similarity with 3 strains in the database GenBank was observed. The first strain *Microbispora* sp. H347 was isolated from sample of Korean soil, and was utilized in a phylogenetic study of the Genus *Microbispora*, however this strain formed a clade with the type stains *M. rosea* and *M. parva* and have no support to classification as a unique species [19]. The others stains correlated are *Microbispora* sp. CRCB5, strain utilized in cellulose- decomposing, but was not characterized on species level [8], and the type strain *Microbispora rosea* subsp. *rosea* JCM8971 [21]. Strain LGMB259 formed a clade with *Microbispora* sp. H347 in the 16S rRNA phylogenetic analysis, this analysis showed conflicting topologies and no support to classification in species level. So we assumed that the 16S rRNA analysis is more appropriate for discrimination on the genus level, and a multigene study is necessary to phylogenetic characterization of this genus.

1-Vinyl-*β*-carboline-3-carboxylic acid (**1**), was responsible for the antibacterial activity of the extract from R5 A-medium, this compound showed high cytotoxic activity and moderate activity against the yeast *S. cerevisiae* ATCC204508, and the phytopathogens *P. citricarpa* LGMB06 and *C. gloeosporioides* FDC83. In contrast, JBIR-133 (**2**) and kitasetaline (**3**) neither revealed biological activity, as well the compound methyl 1-(propionic acid)- β-carboline -3-carboxylic acid (**4**) [17], indicating that the vinyl side chain attached at 1-position in compound **1** is crucial for the biological activities of this natural product. *β*-Carbolines are nitrogen-containing heterocyclic compounds that consists of a pyridine ring fused to an indole skeleton [6]. Only ~30 *β*-carboline derivatives have been reported from bacteria so far [18]. They show an interesting biological activity spectrum, ranging from antibacterial over fungicidal to herbicidal. Some of them were reported as having affinity to the benzodiazepine-receptor [7, 26]. 1-Vinyl-*β*-carboline-3-carboxylic acid (**1**) was reported for the first time as natural product from *Nocardiopsis dassonvillei* (Ichihara, T., Japanese patent application JP 57-169481 A. 1982). However NMR assignments of this natural product based on extensive 1D and 2D NMR analyses and antifungal activity are reported here for the first time. Compounds JBIR-133 (**2**) [3] and kitasetaline (**3**) [2] were recently reported as metabolites of genetically modified *Kitasatospora setae* NBRC 14216 strains. Both compounds are reported here for the first time from a wild type bacterial strain.

Indoles are a group of compounds produced by a big group of organisms, and are ubiquitously present in higher plants and a wide range of microbes particularly those in association with plant [9]. We have isolated indole-3-carbaldehyde (**5**), indole-3-acetic acid (**7**) and indole-3-carboxylic acid (**6**) from strain *Microbispora* LGMB259. Recent findings about indole-3-acetic acid suggest that this compound serves as a signaling molecule in certain

plant-associated microbes and it might exert an indispensable impact in microbe-plant interaction and was correlated with plant growth promotion [20]. Furthermore the compound indole-3-carboxylic acid was related to an active role in the induced resistance upon infection by fungi in plants [13]. These data suggest the possibility of strain LGMB259 be utilized in biological control, since this microorganism produces compounds with antimicrobial activity (including phytopathogens), compounds that improve defense systems in plants and was isolated as an endophytic microbe.

In summary, the strain was characterized as *Microbispora* sp. LGMB259 by morphologic and phylogenetic analysis. This strain produced as activity metabolite 1-vinyl-*β*-carboline-3-carboxylic acid (**1**), a compound that displayed high antibacterial activity against selected Gram-positive bacterial, moderate antifungal activity and considerable cytotoxic activity against PC3 and A549 human cancer cell lines. Overall, these bioactivity studies provide new insights into the structure-activity-relationships (SAR) of *β*-carbolines, and show that the vinyl side chain is essential for the observed biological activities. This research also opens an interesting possibility about the utilization of strain LGMB259 as biological control, due the capacity of survive in the plants tissues and produce compounds with biological interest in the plant defense.

Acknowledgements

We thank Dr. Jack Goodman (University of Kentucky) for the HRESIMS measurements and Dr Francisco André Ossamu Tanaka (NAP/MEPA) for the Scanning Electron Microscope analysis. D. C. S. thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for a short term scholarship. This work was supported in part by the University of Kentucky College of Pharmacy, the University of Kentucky Markey Cancer Center and the National Center for Advancing Translational Sciences (UL1TR000117). This work was also supported by NIH grant CA 91091 to J.R, and Fundação Araucária – Apoio ao desenvolvimento científico e tecnológico do Paraná.

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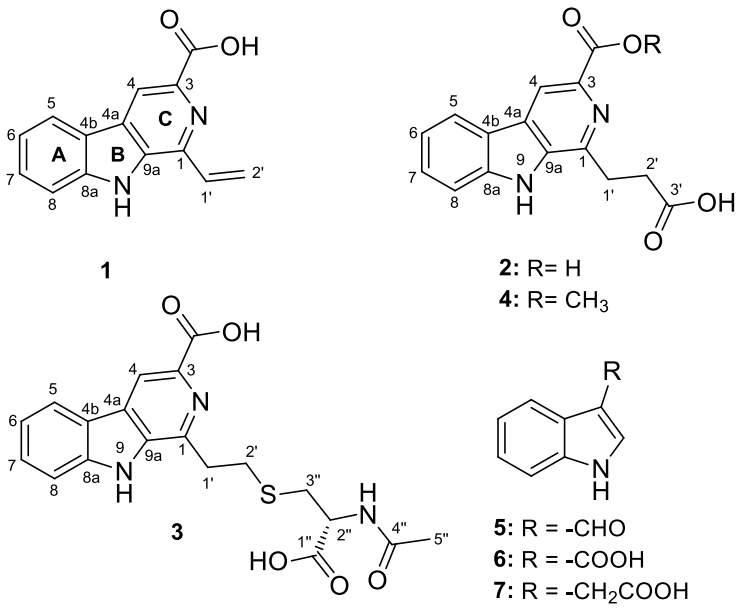

Figure 1 Chemical structure of compounds **1**-**7**

Figure 2 Scanning electron micrograph of spherical paired spores with smooth surfaces of strain *Microbispora* sp. LGMB259 grown on ISP medium 3 for 15 days at 37 ºC. Bar, 10 µm.

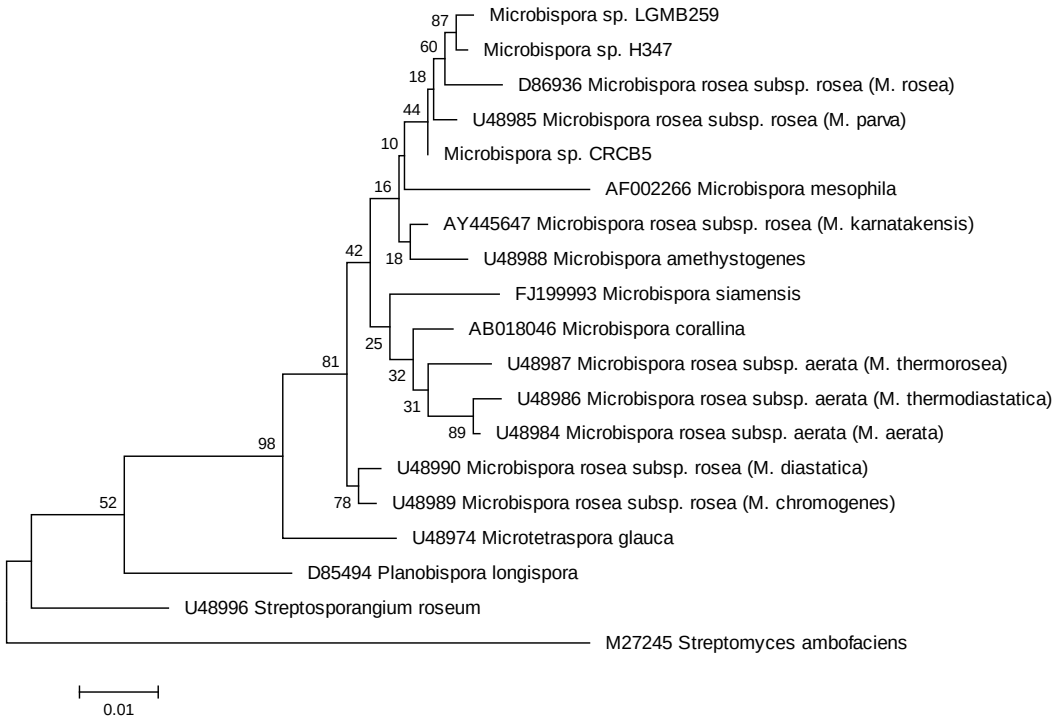

Figure 3 Maximum Likelihood Phylogenetic tree for *Microbispora* genus using 16S rRNA gene. Bootstrap support is shown on nodes. The tree was rooted with *Streptosporangium roseum* (U48996).

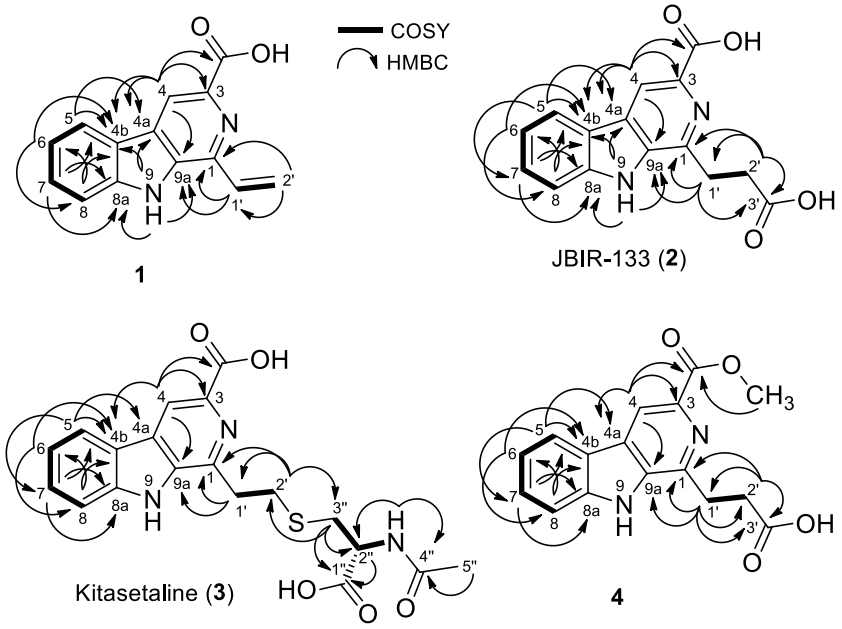

Figure 4 Selected ^{1}H,^{1}H-COSY (▬) and HMBC (→) correlations of *β*-carbolines **1**-**4**

Table 1 Physico-chemical properties of compounds **1-4**

	1 [a]	**2** [a]	**3** [a]	**4** [a]
Molecular Formula	$C_{14}H_{10}N_2O_2$	$C_{15}H_{12}N_2O_4$	$C_{19}H_{19}N_3O_5S$	$C_{16}H_{14}N_2O_4$
Appearance	Pale-yellow solid, UV-absorbing (254 nm)	Pale-yellow solid, UV-absorbing (254 nm)	Pale-yellow solid, UV-absorbing (254 nm)	Pale-yellow solid, UV-absorbing (254 nm)
HPLC-R$_t$	12.86 (min)	12.48 (min)	12.37 (min)	20.06 (min)
R_f	0.28 (DCM/10% MeOH)	0.10 (DCM/20% MeOH)	0.34 (DCM/20% MeOH)	0.13 (DCM/20% MeOH)
Anisaldehyde/H_2SO_4 reagent	Yellow	Yellow	Yellow	Yellow
(+)-APCI-MS: *m/z*	239 $[M + H]^+$, 477 $[2M + H]^+$, 193 $[(M\text{-}COOH) + H]^+$	285 $[M + H]^+$, 267 $[M - H_2O]^+$, 239 $[(M\text{-}COOH) + H]^+$	402 $[M + H]^+$, 273 $[(M\text{-}Cys) + H]^+$	299 $[M + H]^+$, 267 $[M - H_2O]^+$, 239 $[(M\text{-}COOH) + H]^+$
(−)-APCI-MS: *m/z*	237 $[M - H]^-$, 475 $[2M - H]^-$, 191 $[(M\text{-}COOH) - H]^-$	-	400 $[M - H]^-$, 801 $[2M - H]^-$, 271 $[(M\text{-}Cys) - H]^-$	-
(+)-HRESI-MS: *m/z*	239.0814 $[M + H]^+$	-	-	-
Calcd.	239.0815 for $C_{14}H_{11}N_2O_2$ $[M + H]^+$	-	-	-
(−)-HRESI-MS: *m/z*	237.0662 $[M - H]^-$	-	-	-
Calcd.	237.0669 for $C_{14}H_9N_2O_2$ $[M - H]^-$	-	-	-
UV/VIS (MeOH): λ_{max} (log ε)	394 sh (3.08), 366 sh (3.42), 282 (4.30), 213 (4.17) nm	374, 278, 240, 213 nm	375, 278, 241, 214 nm	374, 272, 240, 218 nm

a) For HPLC, UV and Mass spectrometry data, see supporting information Figures S4-7, S13, S19 and S25.

Table 2 ^{13}C and ^{1}H NMR data of compounds **1-4** in DMSO-d_6 (mult., *J* in [Hz])

No.	**1** [a]		**2** [a]		**3** [a]		**4** [a]	
	δ_C [b]	δ_H [c]	δ_C [b]	δ_H [c]	δ_C [b]	δ_H [c]	δ_C [b]	δ_H [c]
1	138.3 Cq	-	143.8 Cq	-	142.9 Cq	-	144.6 Cq	-
3	136.5 Cq	-	134.0 Cq	-	134.2 Cq	-	135.8 Cq	-
3-CO	166.4 Cq	-	165.3 Cq	-	165.2 Cq	-	166.1 Cq	-
3CO$\underline{CH_3}$	-	-	-	-	-	-	51.9 CH_3	3.90 (s)
4	116.4 CH	8.85 (s)	116.5 CH	8.93 (s)	116.9 CH	9.02 (s)	116.2 CH	8.79 (s)
4a	129.3 Cq	-	128.7 Cq	-	129.0 Cq	-	128.4 Cq	-
4b	121.0 Cq	-	121.1 Cq	-	121.1 Cq	-	121.3 Cq	-
5	122.2 CH	8.39 (d, 8.0)	122.7 CH	8.44 (d, 8.0)	122.9 CH	8.49 (d, 8.0)	122.1 CH	8.37 (d, 7.5)
6	120.4 CH	7.33 (ddd, 8.0, 8.0, 1.0)	120.9 CH	7.36 (t, 8.0)	121.0 CH	7.39 (t, 8.0)	120.2 CH	7.30 (t, 8.0)
7	128.9 CH	7.63 (ddd, 8.0, 7.5, 1.5)	129.6 CH	7.66 (t, 8.5)	130.1 CH	7.70 (t, 8.0)	128.4 CH	7.59 (t, 7.0)
8	112.4 CH	7.67 (dd, 7.5, 1.0)	112.7 CH	7.71 (d, 8.5)	112.8 CH	7.74 (d, 8.5)	112.4 CH	7.66 (d, 8.5)
8a	141.3 Cq	-	141.8 Cq	-	142.1 Cq	-	140.8 Cq	-
9	-	12.22 (s)	-	12.54 (brs)	-	12.67 (brs)	-	12.20 (brs)
9a	134.9 Cq	-	135.6 Cq	-	135.6 Cq	-	135.8 Cq	-
1'	131.1 CH	7.46 (dd, 17.0, 10.5)	27.1 CH_2	3.50 (t, 7.5)	32.0 CH_2	3.57 (t, 7.5)	28.5 CH_2	3.38 (t, 7.5)
2'	120.7 CH_2	6.68 (dd, 17.0, 1.5, H_a) 5.77 (dd, 11.0, 1.5, H_b)	31.8 CH_2	2.91 (t, 7.5)	30.3 CH_2	3.09 (t, 7.5)	32.0 CH_2	2.87 (t, 8.0)
3'	-	-	173.9 Cq	-	-	-	174.2 Cq	-
N-Acetyl-L-Cys								
1"	-	-	-	-	172.3 Cq	-	-	-
2"	-	-	-	-	52.1 CH	4.44 (m)	-	-
2"-NH	-	-	-	-	-	8.30 (d, 8.5)	-	-
3"	-	-	-	-	33.1 CH_2	3.03 (dd, 13.5, 5.0) 2.86 (dd, 13.5, 8.5)	-	-
4"	-	-	-	-	169.5 Cq	-	-	-
5"	-	-	-	-	22.4 CH_3	1.84 (s)	-	-

[a] Supporting information Figures S8-12, S14-18, S20-24 and S26-30for the NMR spectra; [b] 125 MHz; [c] 500 MHz;

Table 3 Inhibition halo (in millimeters) of LGMB259 crude extracts of different culture medium and compounds **1**-**3** tested antibacterial and antifungal activities at 100 μg/disc

Extracts /Isolated compounds	***Kocuria rosea***	***Micrococcus luteus***	***Escherichia coli***	***Phyllosticta citricarpa***	***Colletotricum gloesporioides***	***Saccharomices cereviseae***
R5A-medium	33	30	---	NE	NE	NE
SG-medium	---	---	---	---	---	---
M2-medium	---	---	---	---	---	---
Compound **1**	31	30	---	9	17	16
Compound **2**	---	---	---	---	---	---
Compound **3**	---	---	---	---	---	---
Control	30	31	35	27	25	50

--- denotes no measurable halo, NE not evaluated antibacterial control: Ampicillin (1 mg/disc), Antifungal control: amphotericin B (1 mg/disc)

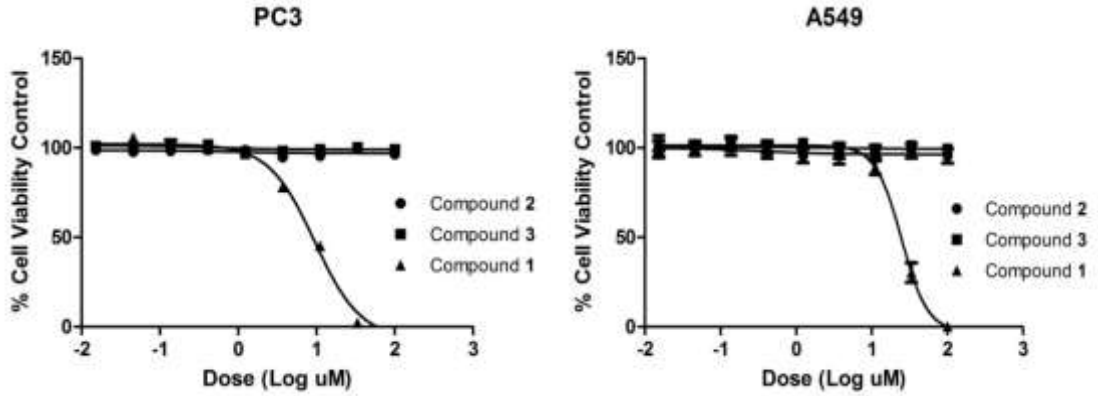

Figure 5 Dose response curve of β-carbolines **1-3**, in PC3 and A549 cell lines at 48h in viability assay.

Supplementary Information

***Microbispora* sp. LGMB259 Endophytic Actinomycete Isolated from *V. divergens* (Pantanal, Brazil) Producing *β*-Carbolines and Indoles with Biological Activity**

Daiani C. Savi [a,b,†], Khaled A. Shaaban[a,c,†], Nathalia Vargas[d], Larissa V. Ponomareva[a,c], Yvelise M. Possiede[e], Jon S. Thorson[a,c], Chirlei Glienke[d#] and Jürgen Rohr[a#]

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0596, U.S.A.[a]; Department of Microbiology, Parasitology and Pathology, Universidade Federal do Parana, Av. Coronel Francisco Heráclito dos Santos, 210. CEP: 81531-970, Curitiba, PR, Brazil[b]; Center for Pharmaceutical Research and Innovation (CPRI), College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0596, U.S.A.[c]; Department of Genetics, Universidade Federal do Parana, PO.BOX 19071. CEP: 81531-970, Curitiba, PR, Brazil[d]; Department of Biology, Universidade Federal do Mato Grosso do Sul, CEP: 79070-900, Campo Grande, MS, Brazil[e].

[†] Authors contributed equally to this work.

Correspondencing authors: Chirlei Glienke, +5533611562, chglienke@gmail.com; Jürgen Rohr, +1 859 257 7564, jrohr2@email.uky.edu

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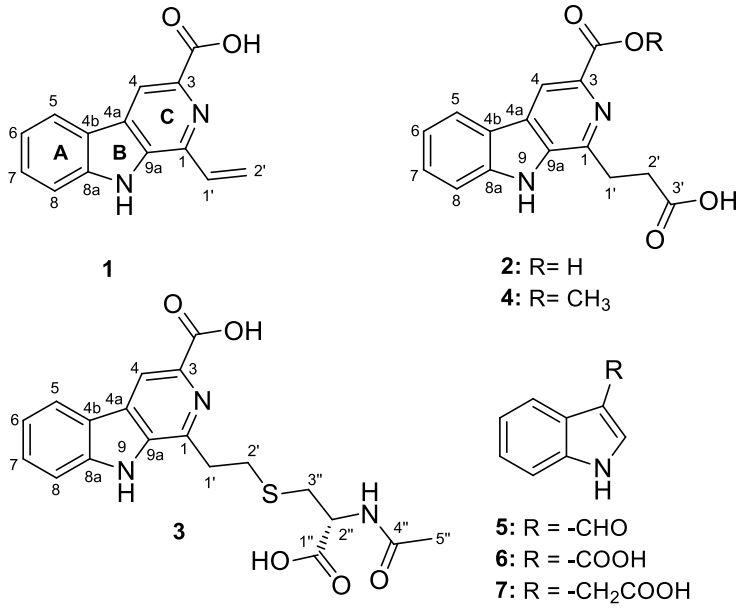

Figure S1: Chemical structures of compounds **1**-**7**

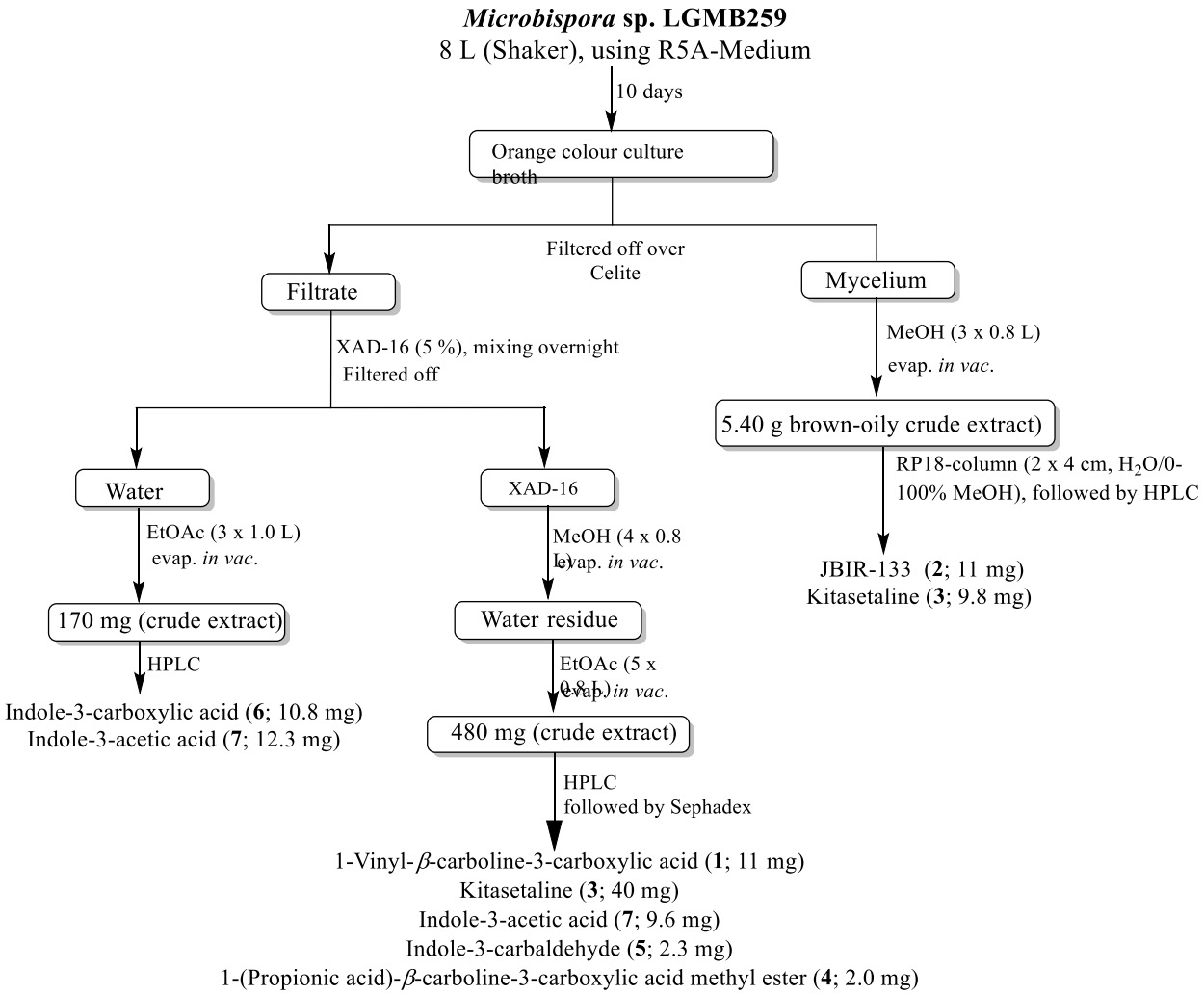

Figure S2: Work-up scheme of *Microbiospora* sp. LGMB259 using R5A-medium

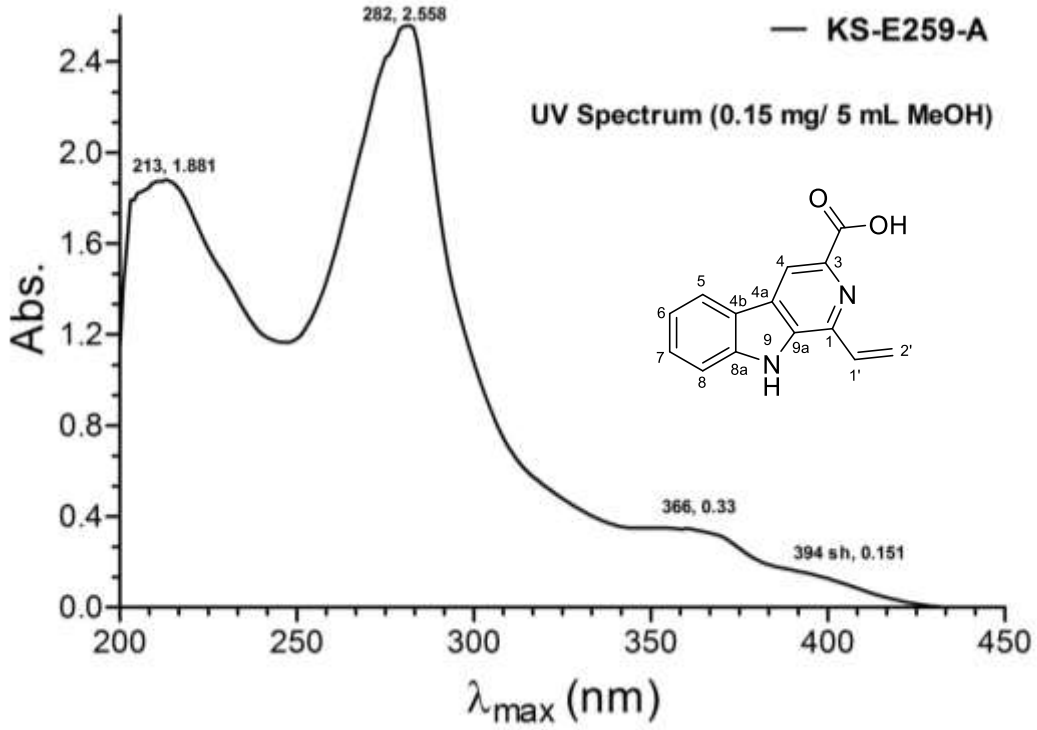

Figure S3: UV (MeOH) spectrum of 1-Vinyl-*β*-carboline-3-carboxylic acid (**1**)

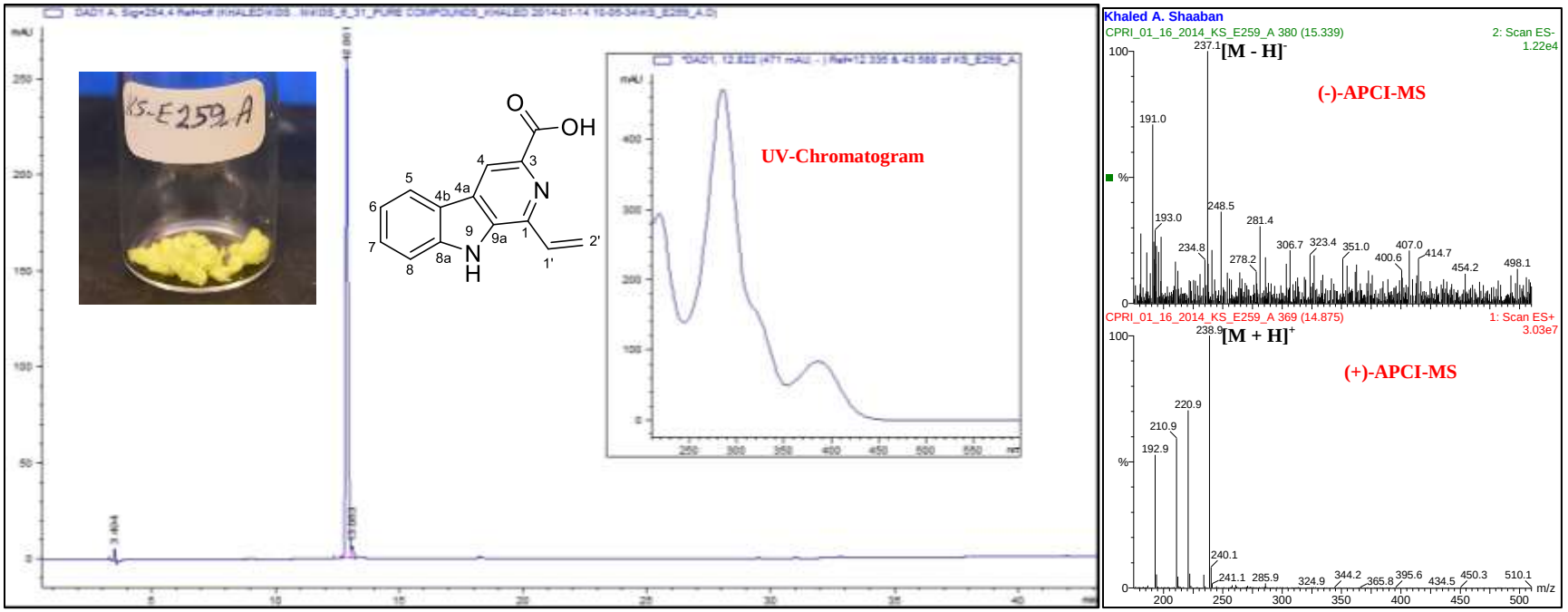

Figure S4: HPLC/UV/APCI-MS analyses of 1-Vinyl-*β*-carboline-3-carboxylic acid (**1**). HPLC-conditions: Detection wavelength 254 nm; solvent A: H_2O/0.1% TFA; solvent B: acetonitrile; flow rate: 1.0 mL min^{-1}; 0-35 min, 95-0% A (linear gradient); 35-40 min 0% A; 40-41 min 0-95% A (linear gradient); 41-45 min 95% A.

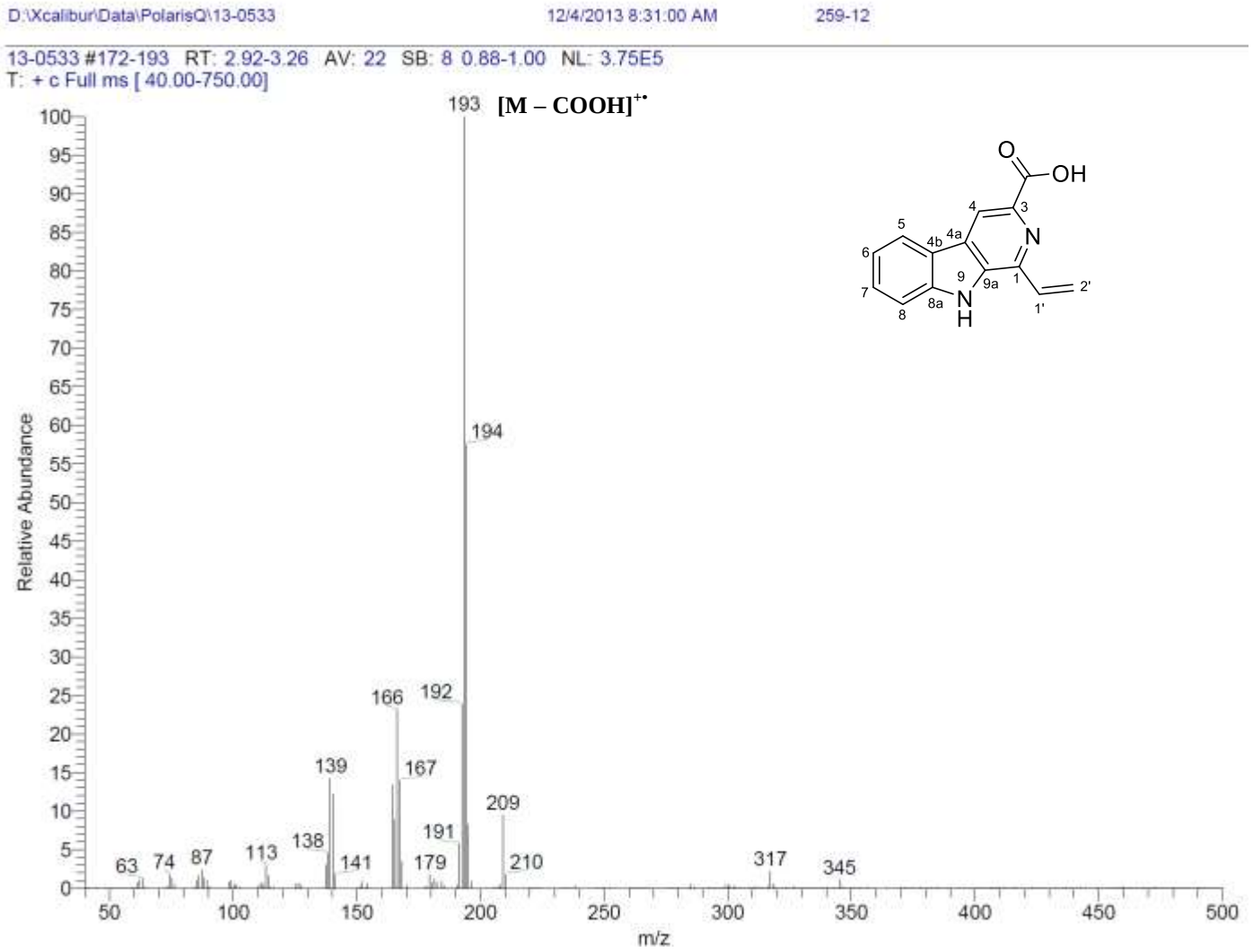

Figure S5: EI-MS spectrum of 1-Vinyl-*β*-carboline-3-carboxylic acid (**1**)

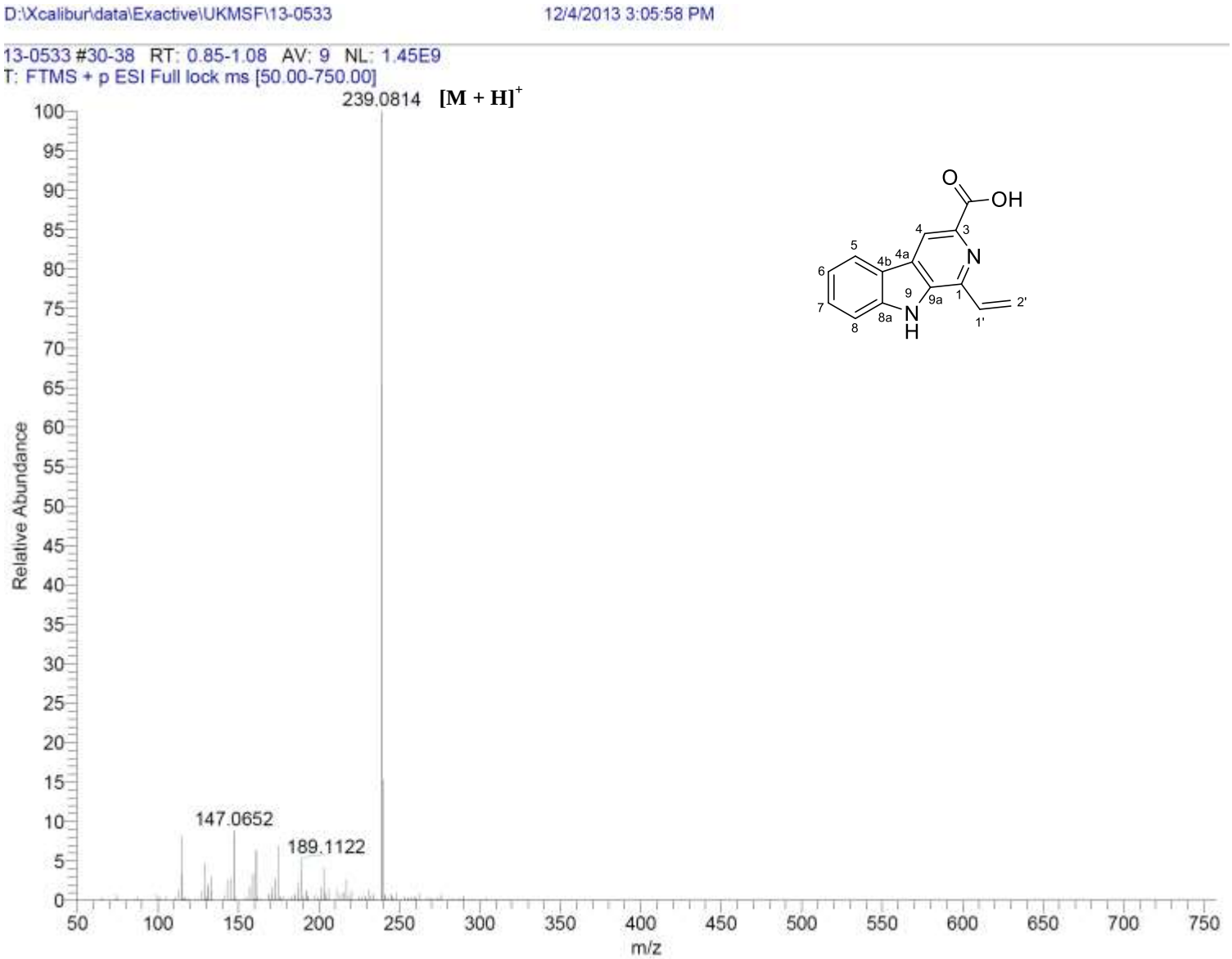

Figure S6: (+)-HRESI-MS spectrum of 1-Vinyl-*β*-carboline-3-carboxylic acid (**1**)

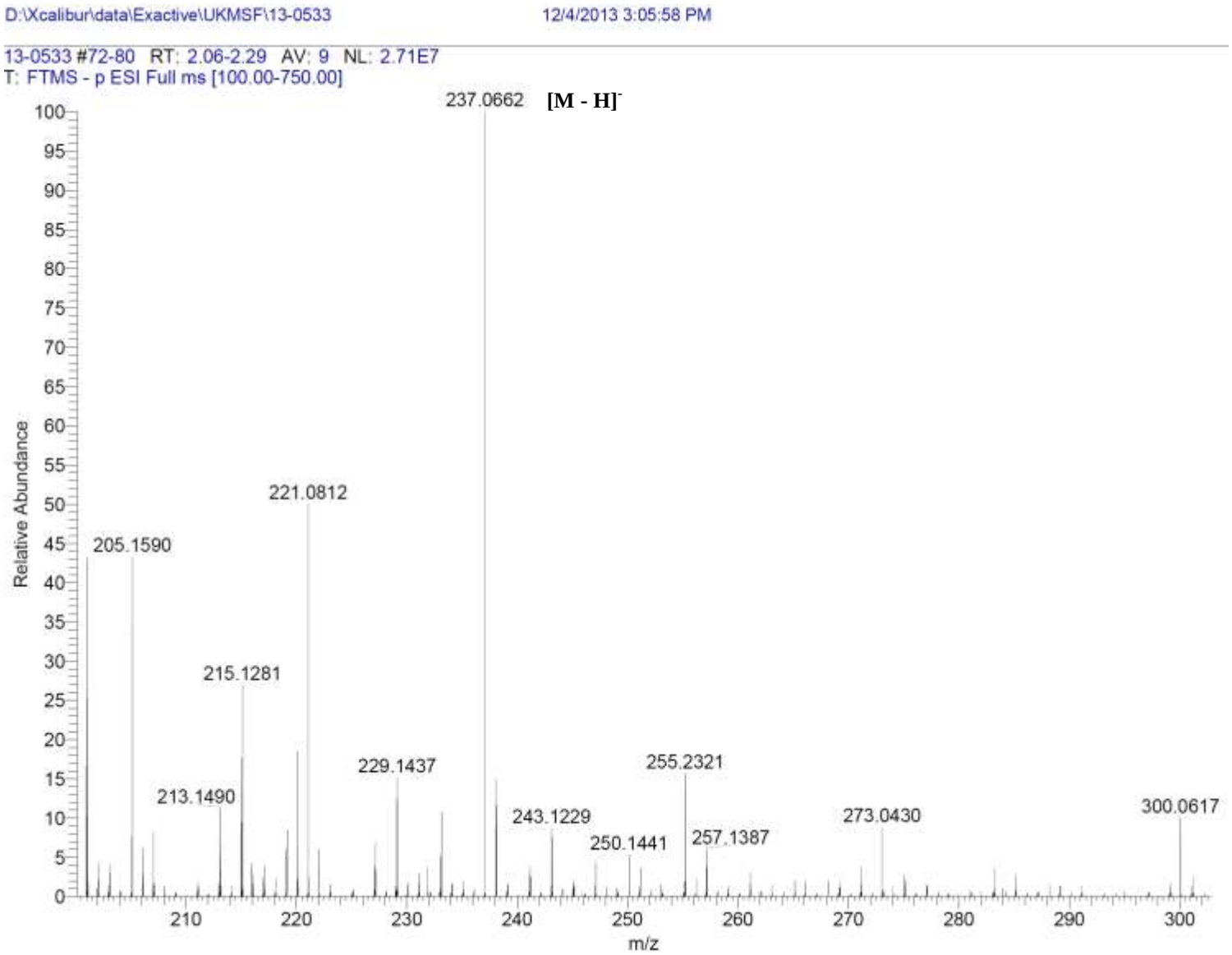

Figure S7: (−)-HRESI-MS spectrum of 1-Vinyl-*β*-carboline-3-carboxylic acid (**1**)

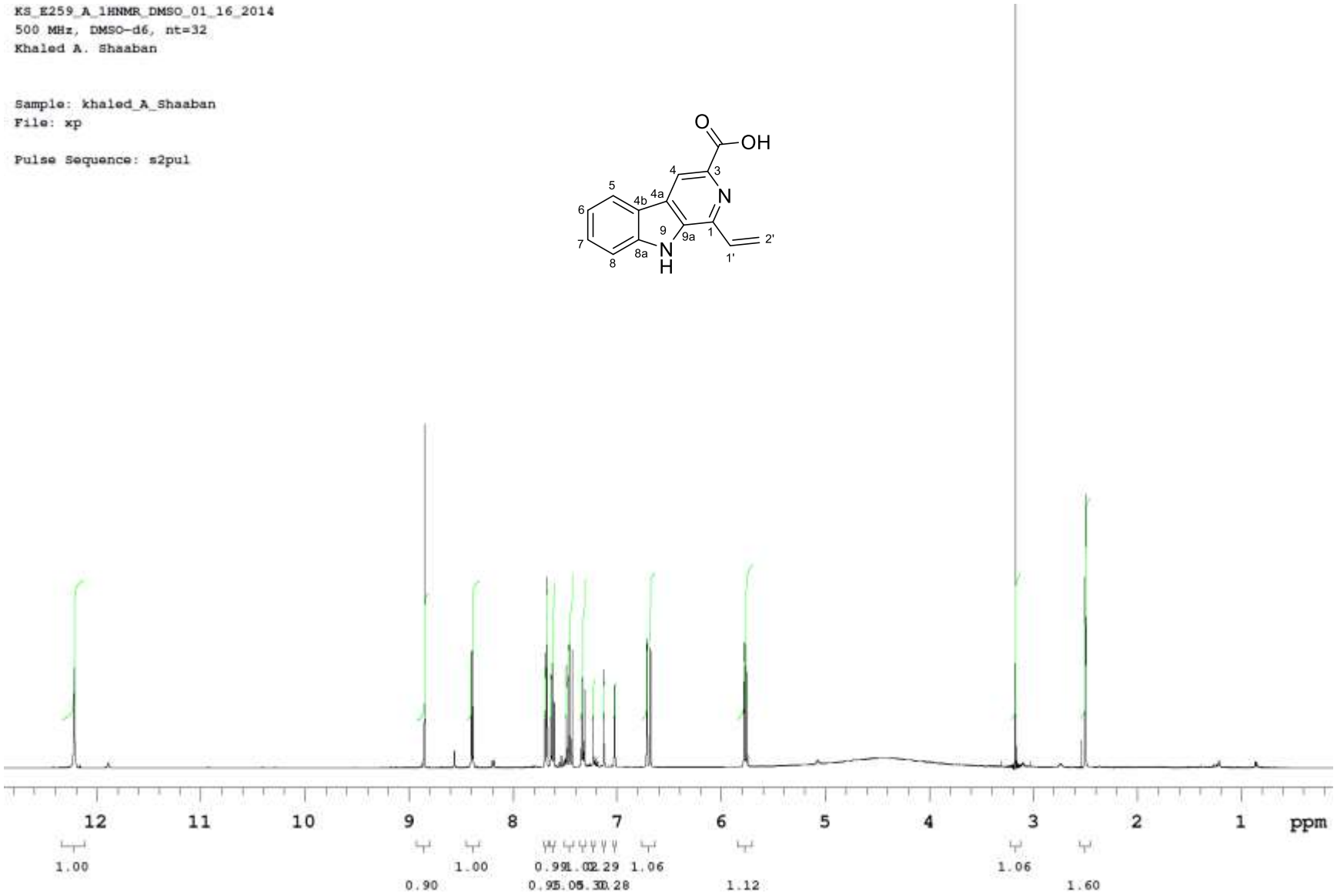

Figure S8: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of 1-Vinyl-β-carboline-3-carboxylic acid (**1**)

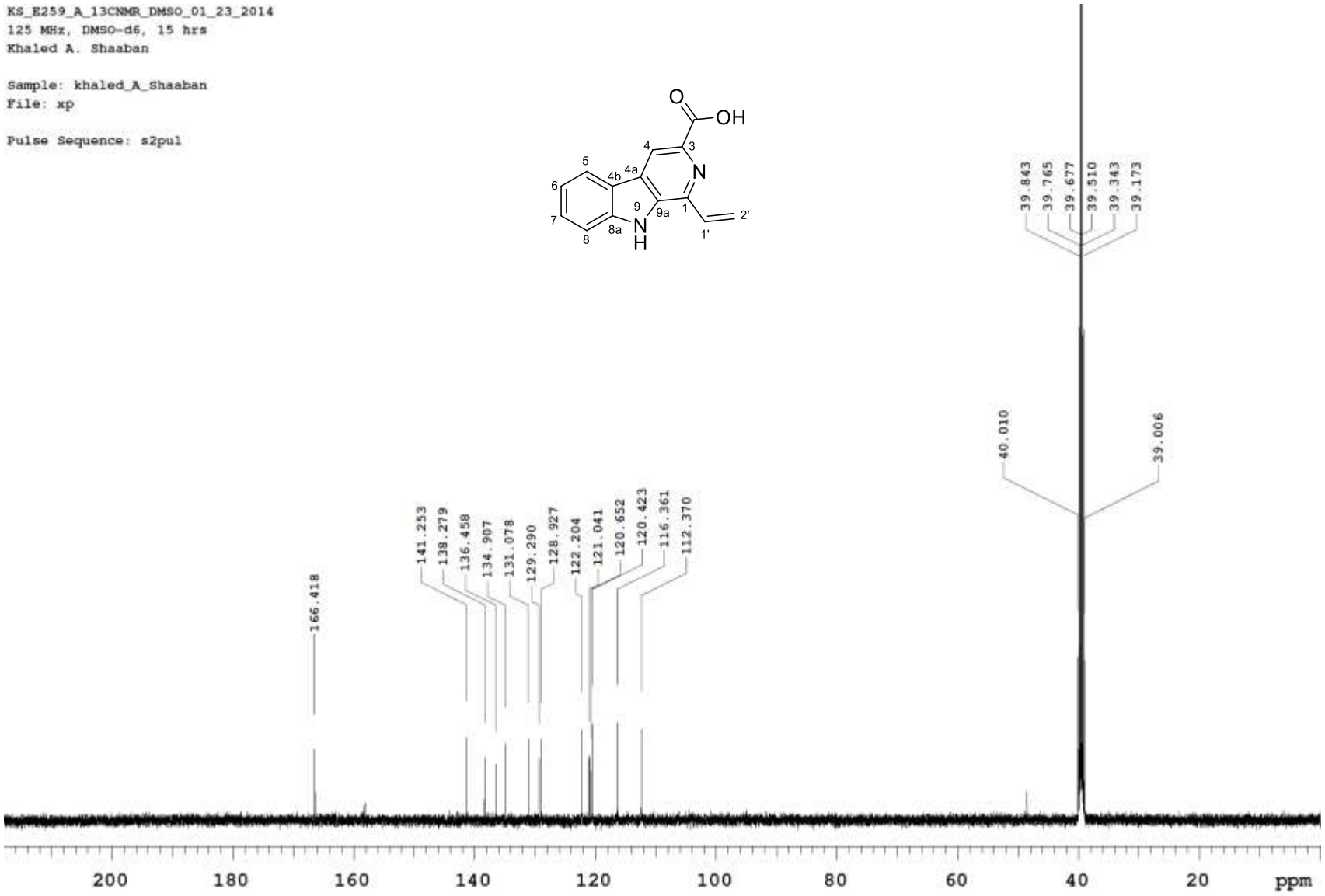

Figure S9: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of 1-Vinyl-β-carboline-3-carboxylic acid (**1**)

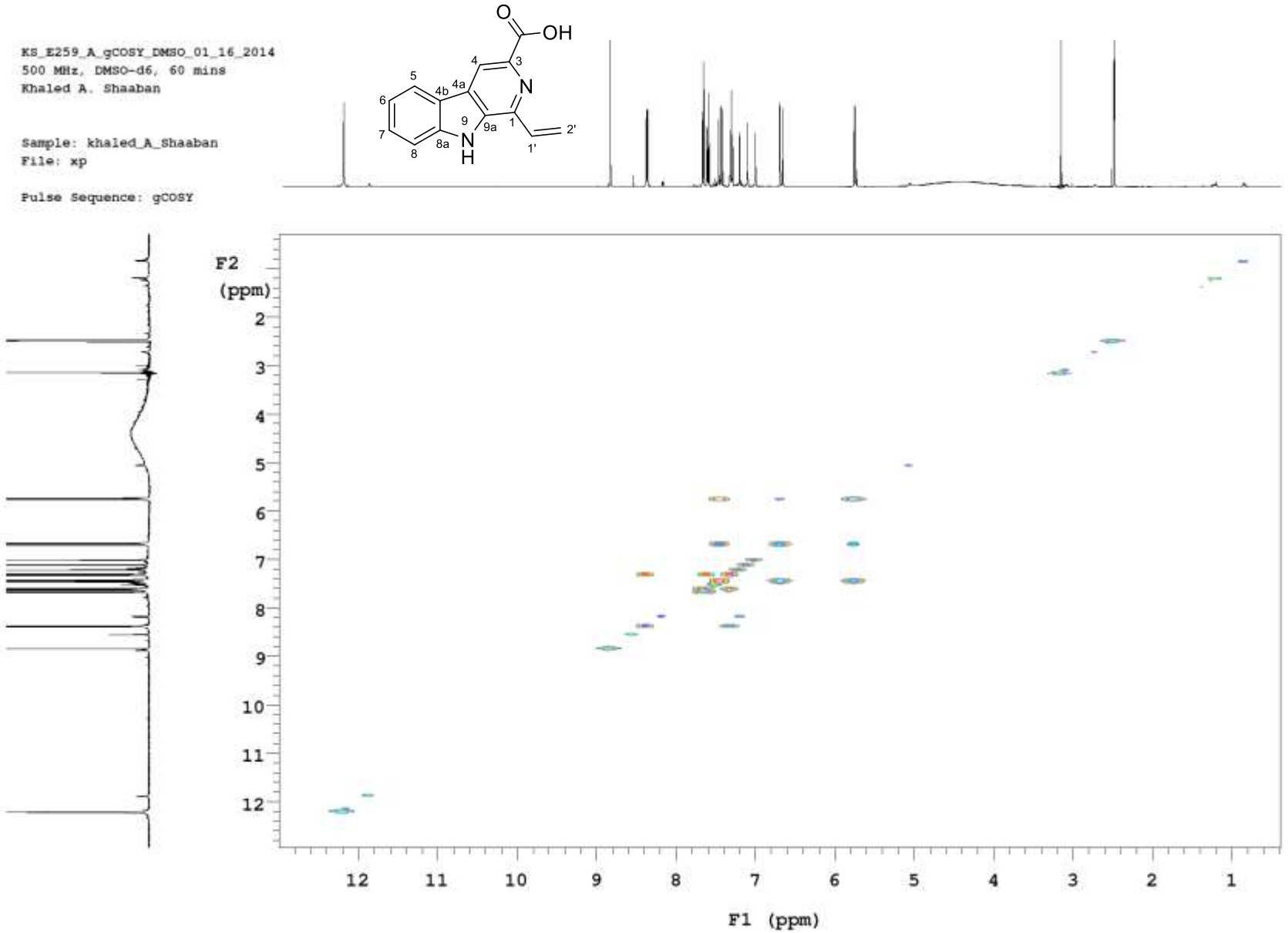

Figure S10: ^{1}H-^{1}H COSY spectrum (DMSO-d_6, 500 MHz) of 1-Vinyl-β-carboline-3-carboxylic acid (**1**)

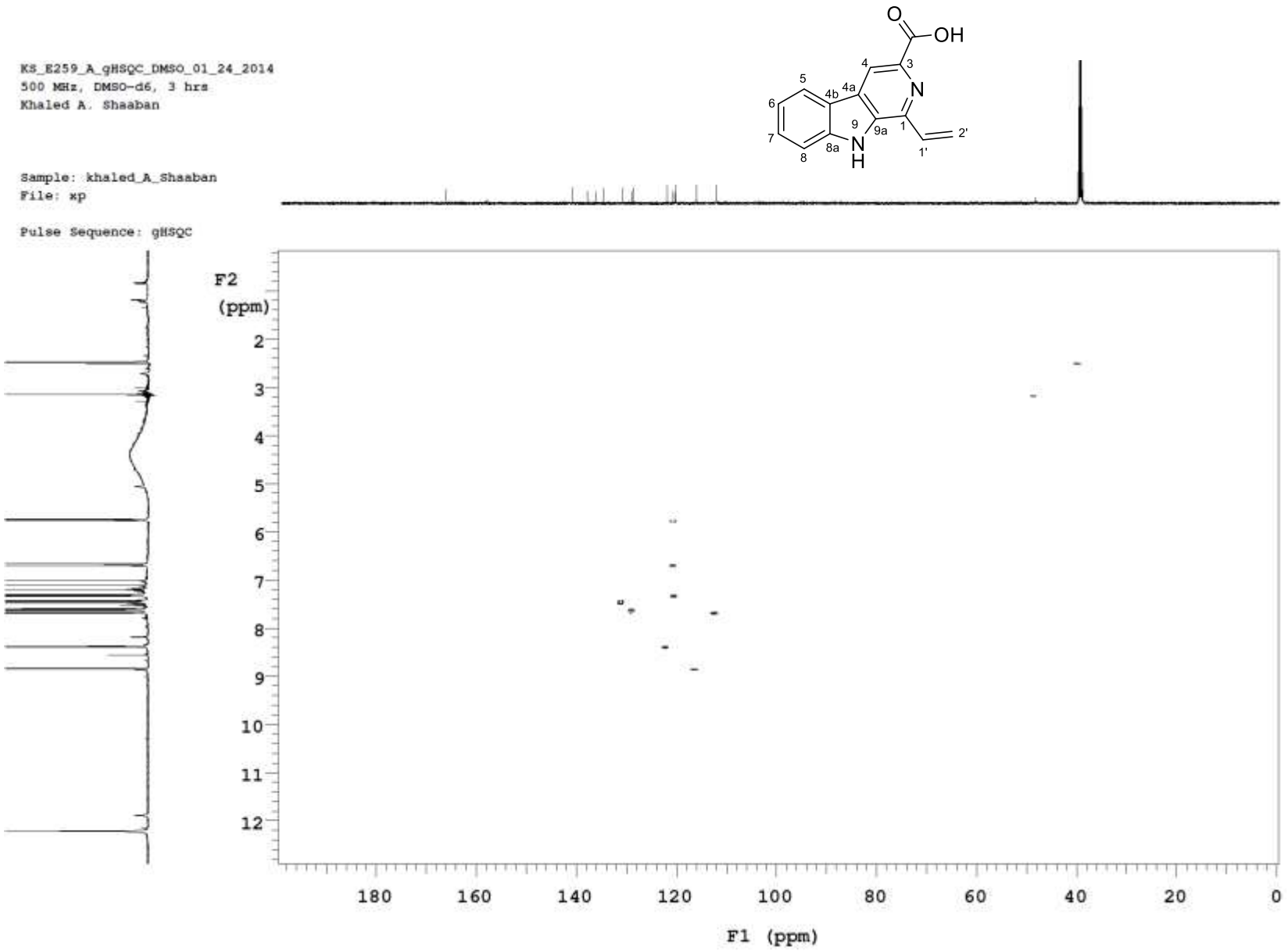

Figure S11: HSQC spectrum (DMSO-d_6, 500 MHz) of 1-Vinyl-β-carboline-3-carboxylic acid (**1**)

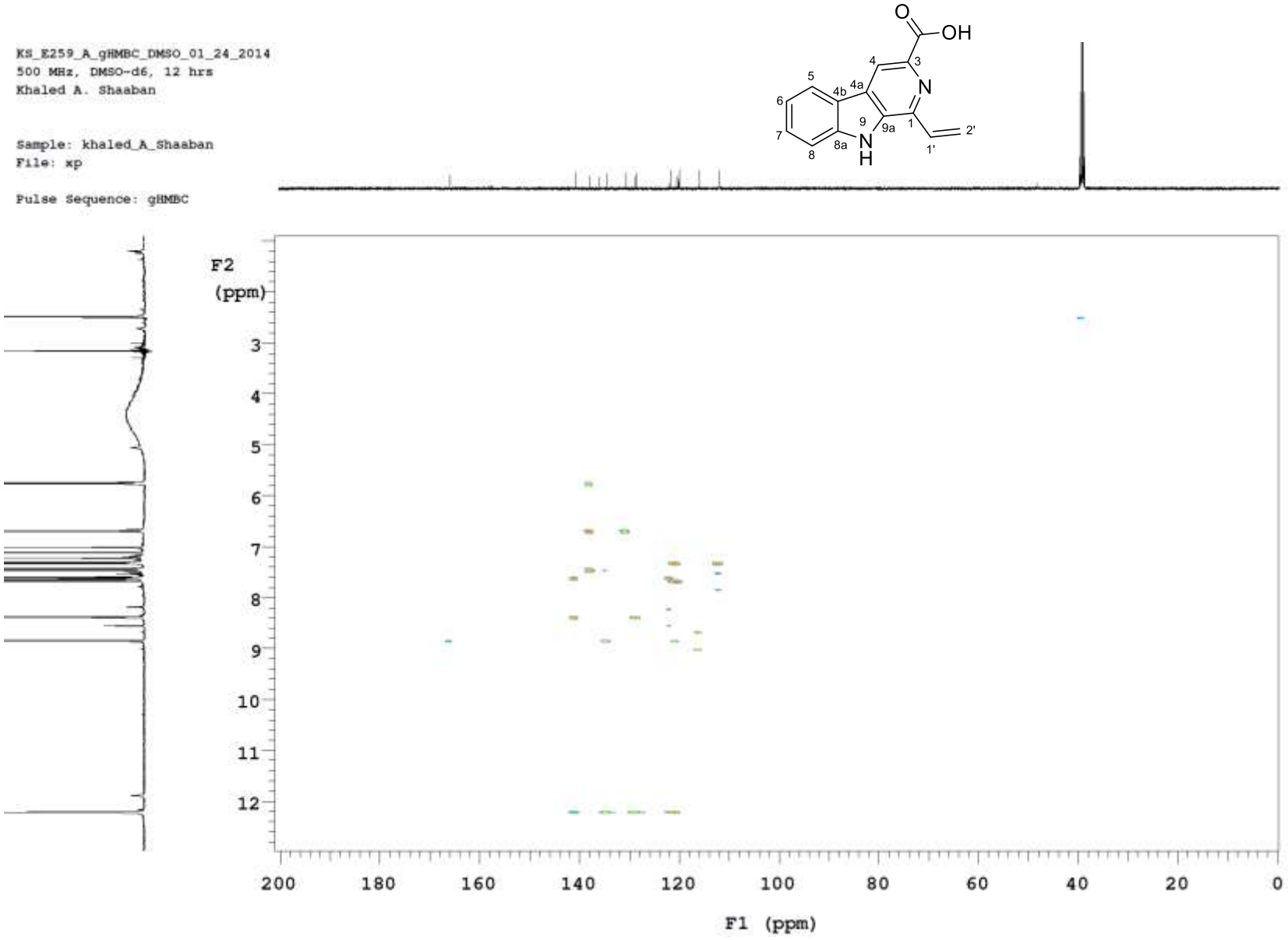

Figure S12: HMBC spectrum (DMSO-d_6, 500 MHz) of 1-Vinyl-β-carboline-3-carboxylic acid (**1**)

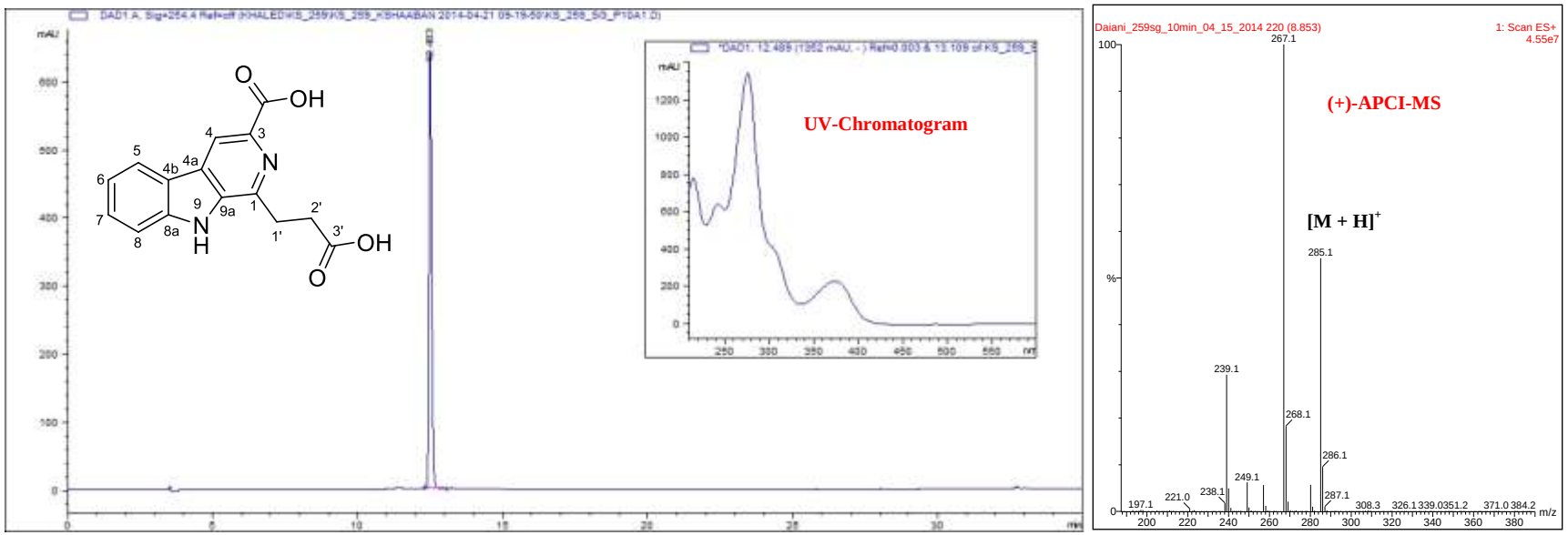

Figure S13: HPLC/UV/APCI-MS analyses of JBIR-133 (**2**). HPLC-conditions: Detection wavelength 254 nm; solvent A: H_2O/0.1% Formic acid; solvent B: acetonitrile; flow rate: 1.0 mL min^{-1}; 0-35 min, 95-0% A (linear gradient); 35-40 min 0-95% A (linear gradient).

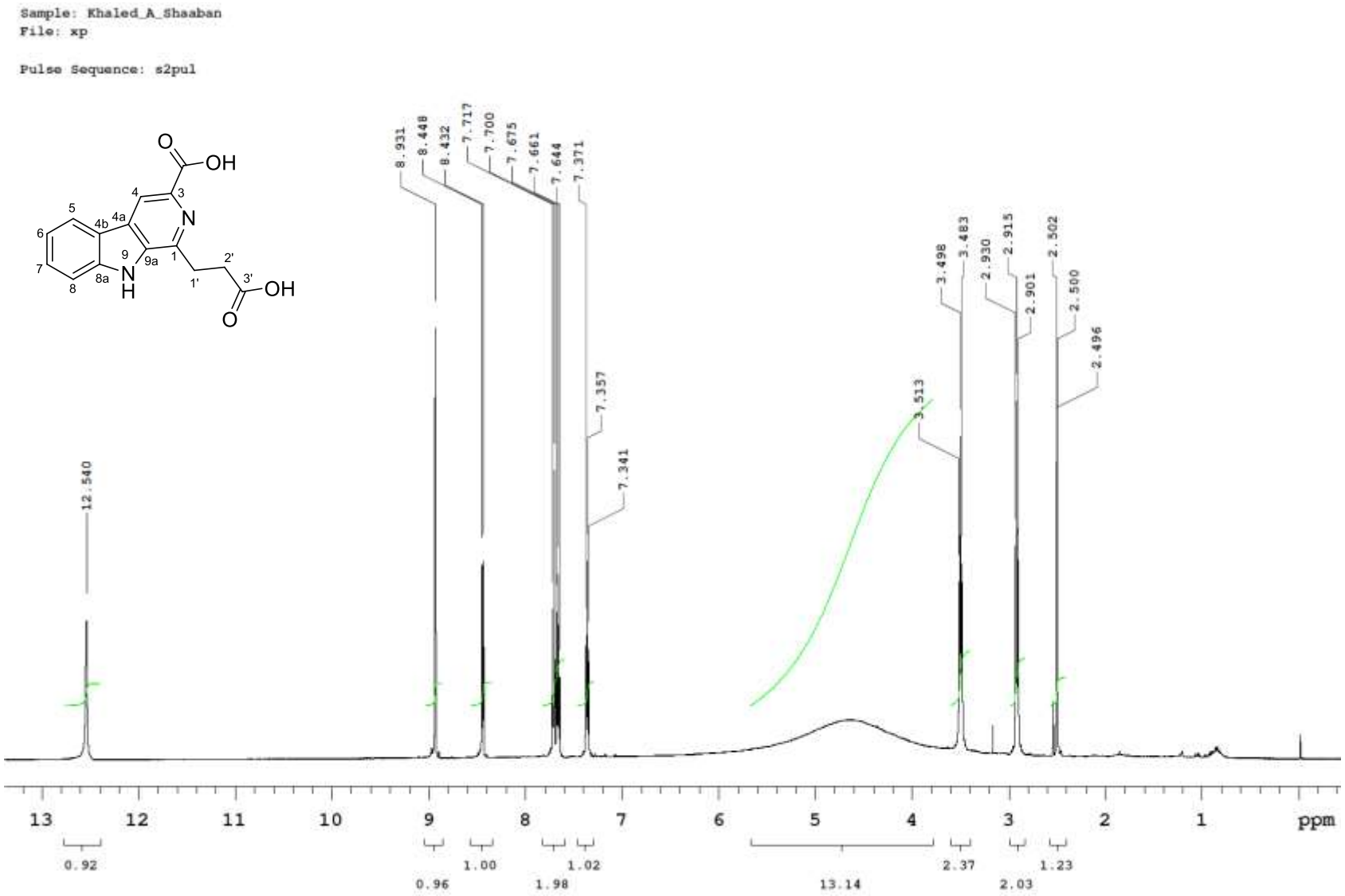

Figure S14: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of JBIR-133 (**2**)

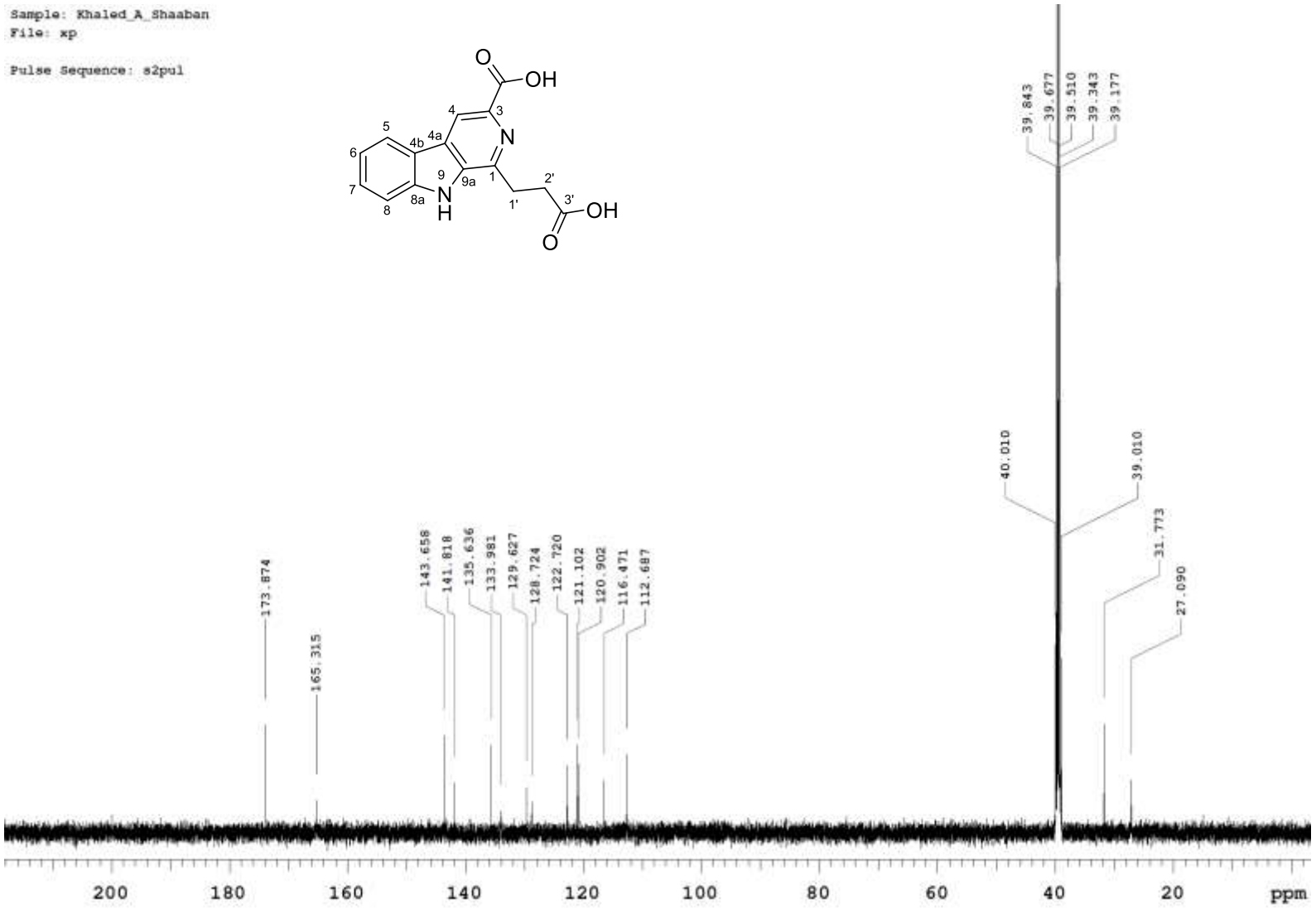

Figure S15: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of JBIR-133 (**2**)

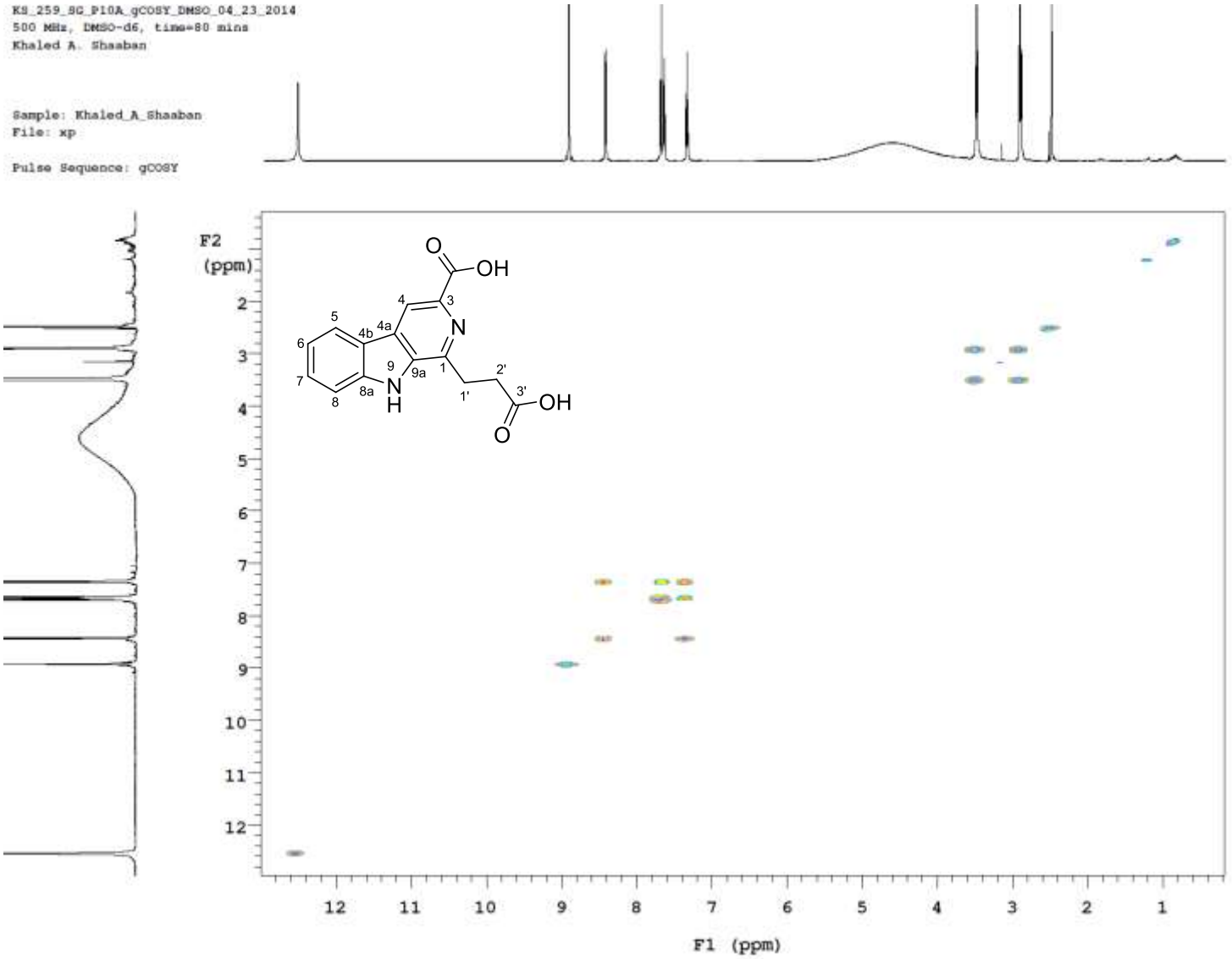

Figure S16: ^{1}H-^{1}H COSY spectrum (DMSO-d_6, 500 MHz) of JBIR-133 (**2**)

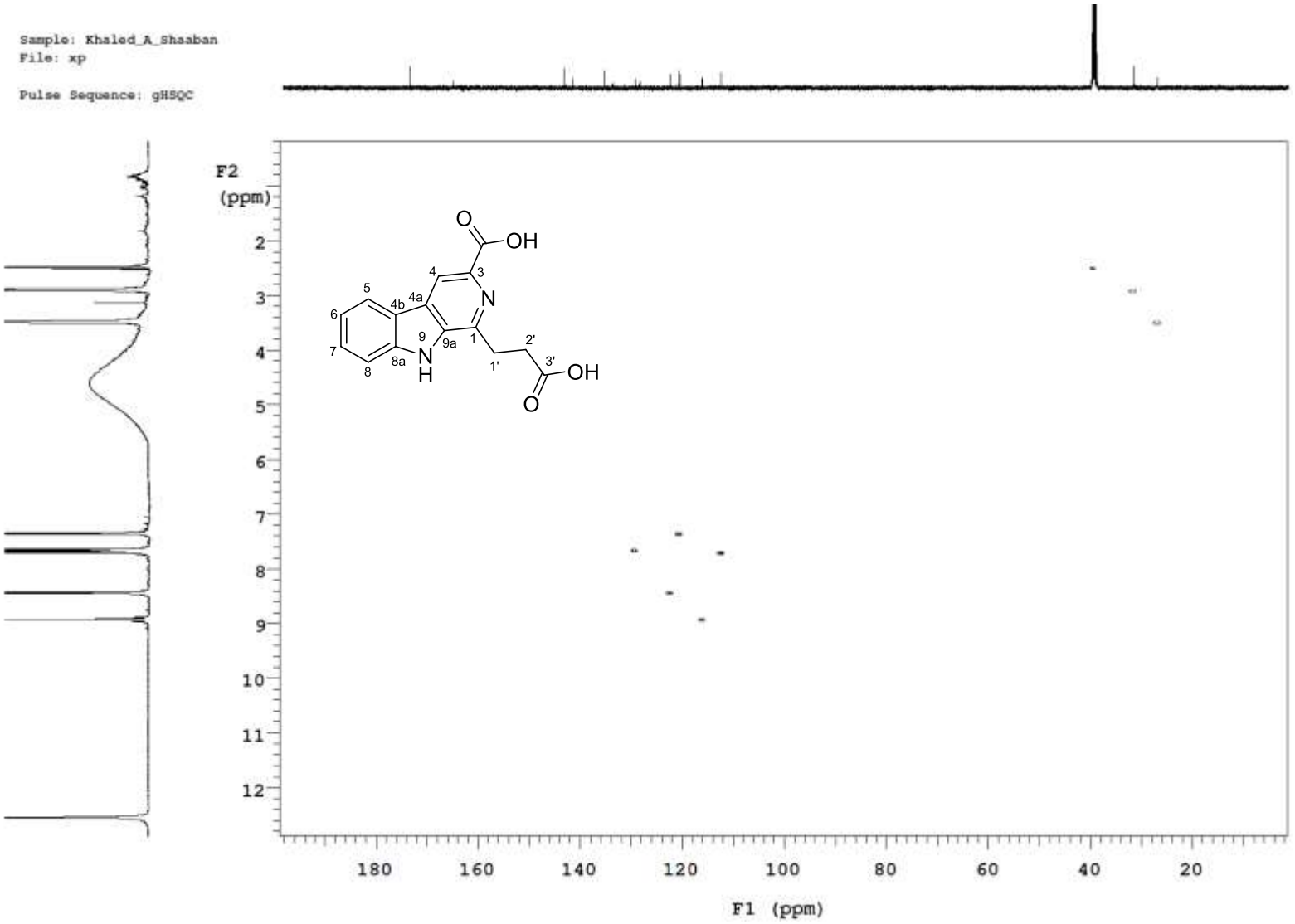

Figure S17: HSQC spectrum (DMSO-d_6, 500 MHz) of JBIR-133 (**2**)

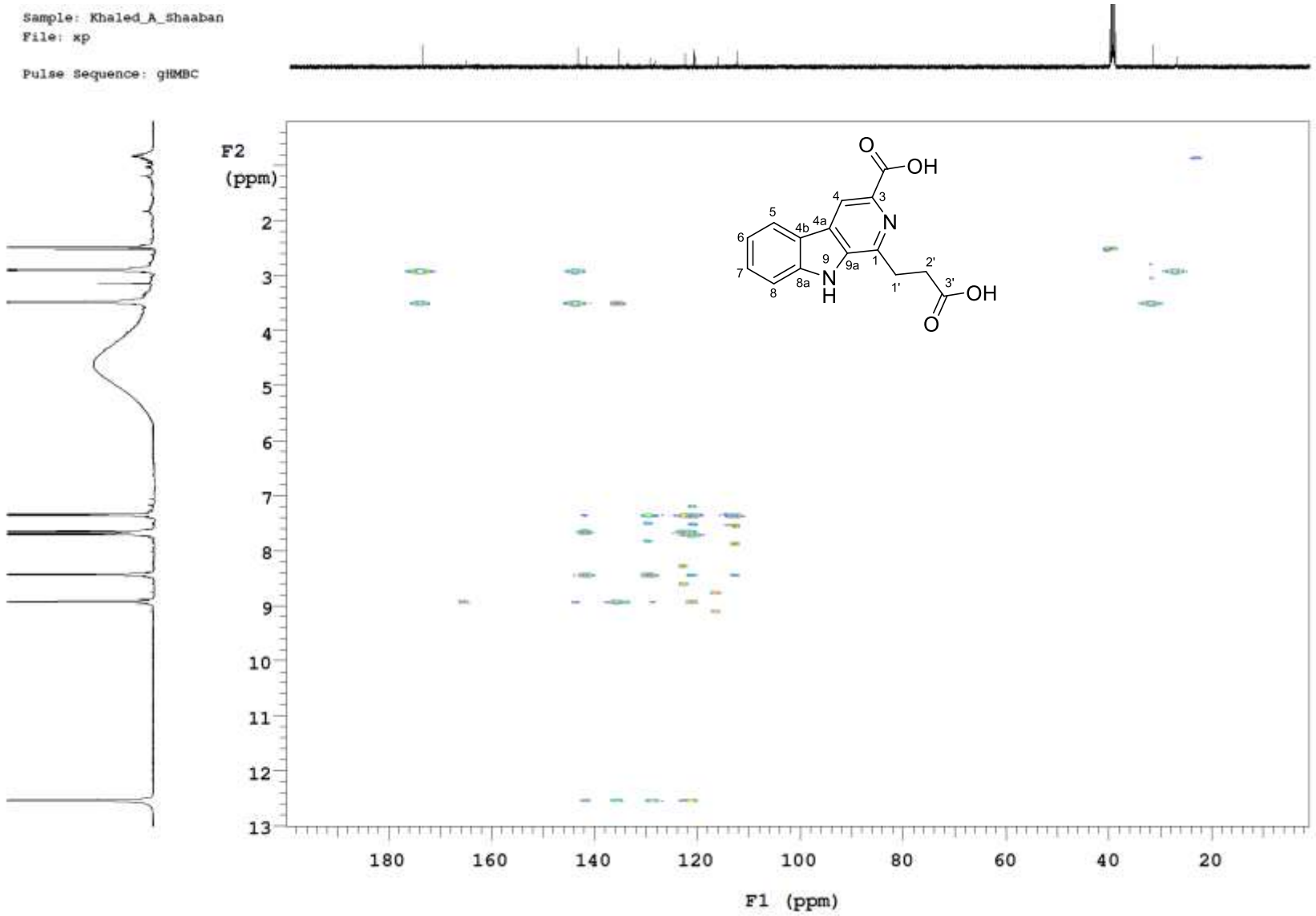

Figure S18: HMBC spectrum (DMSO-d_6, 500 MHz) of JBIR-133 (**2**)

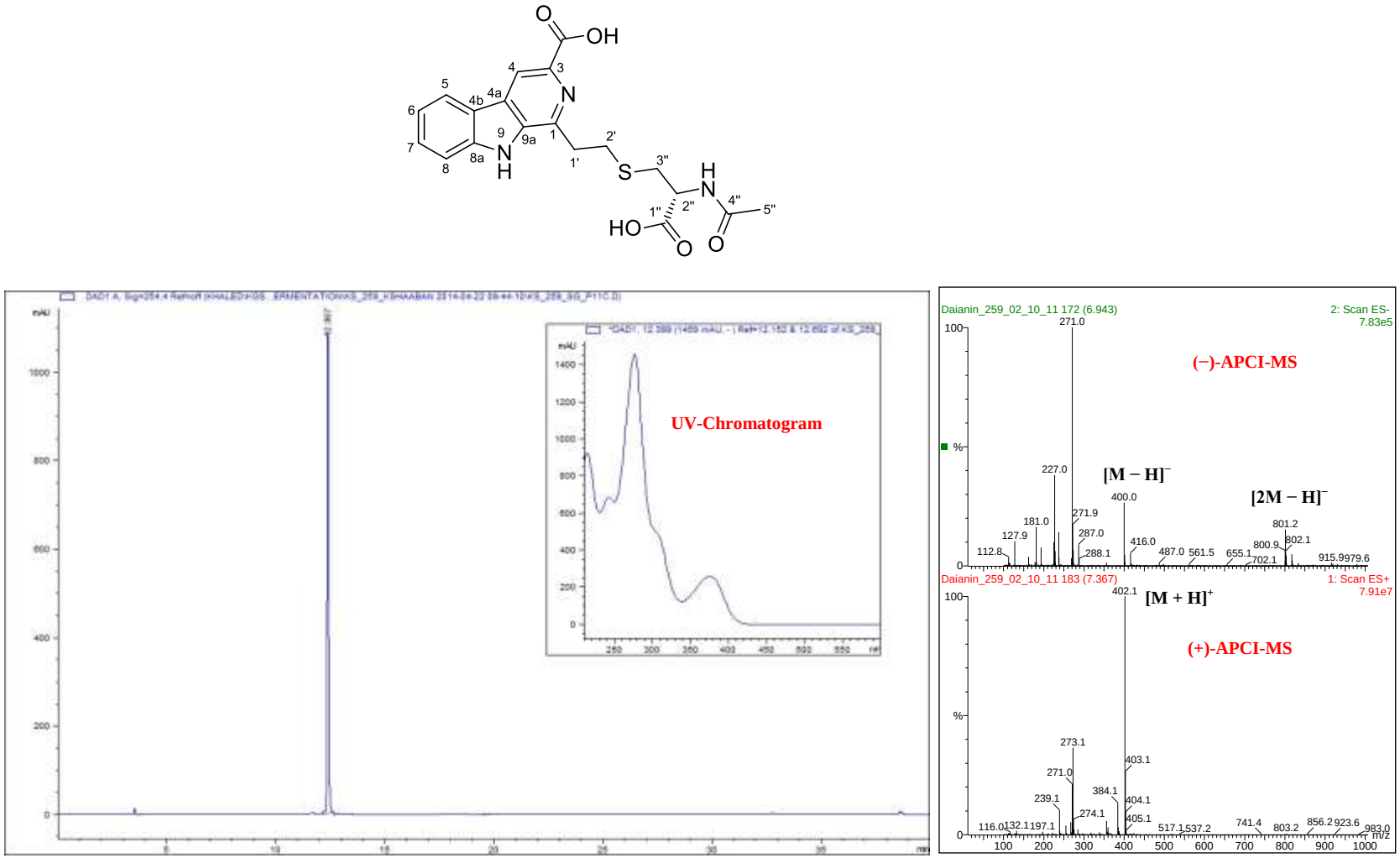

Figure S19: HPLC/UV/APCI-MS analyses of Kitasetaline (**3**). HPLC-conditions: Detection wavelength 254 nm; solvent A: H_2O/0.1% Formic acid; solvent B: acetonitrile; flow rate: 1.0 mL min^{-1}; 0-35 min, 95-0% A (linear gradient); 35-40 min 0-95% A (linear gradient).

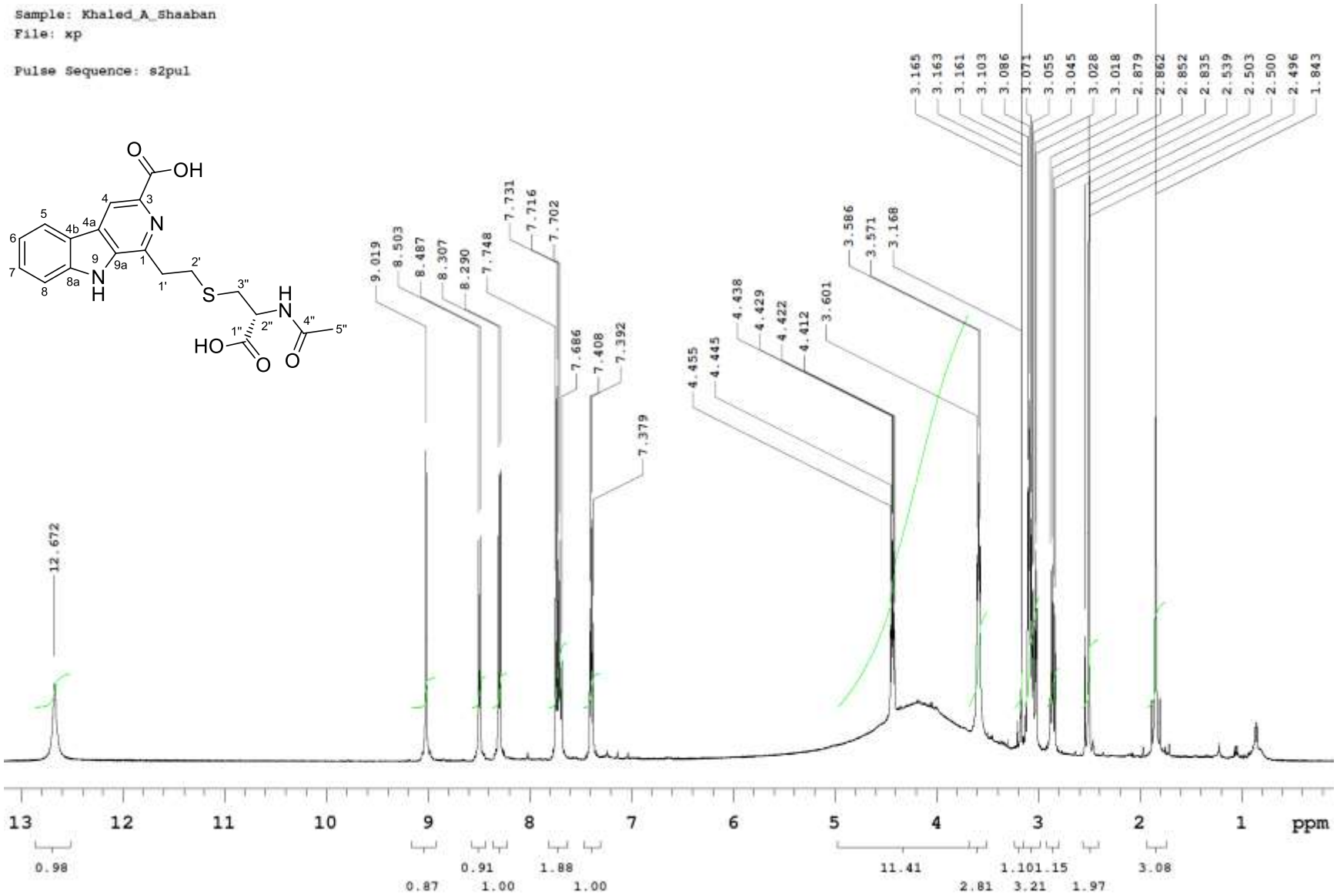

Figure S20: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of Kitasetaline (**3**)

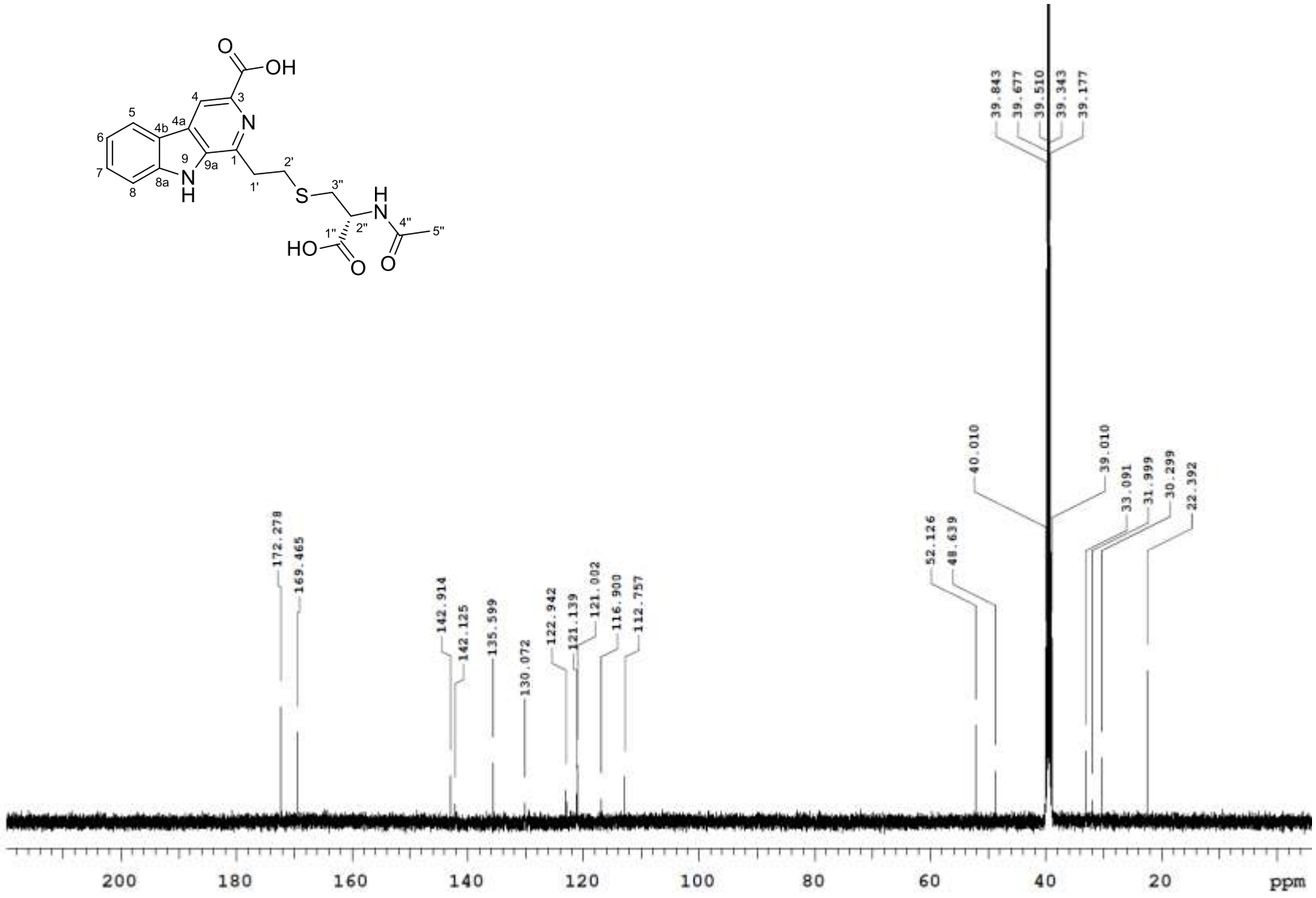

Figure S21: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of Kitasetaline (**3**)

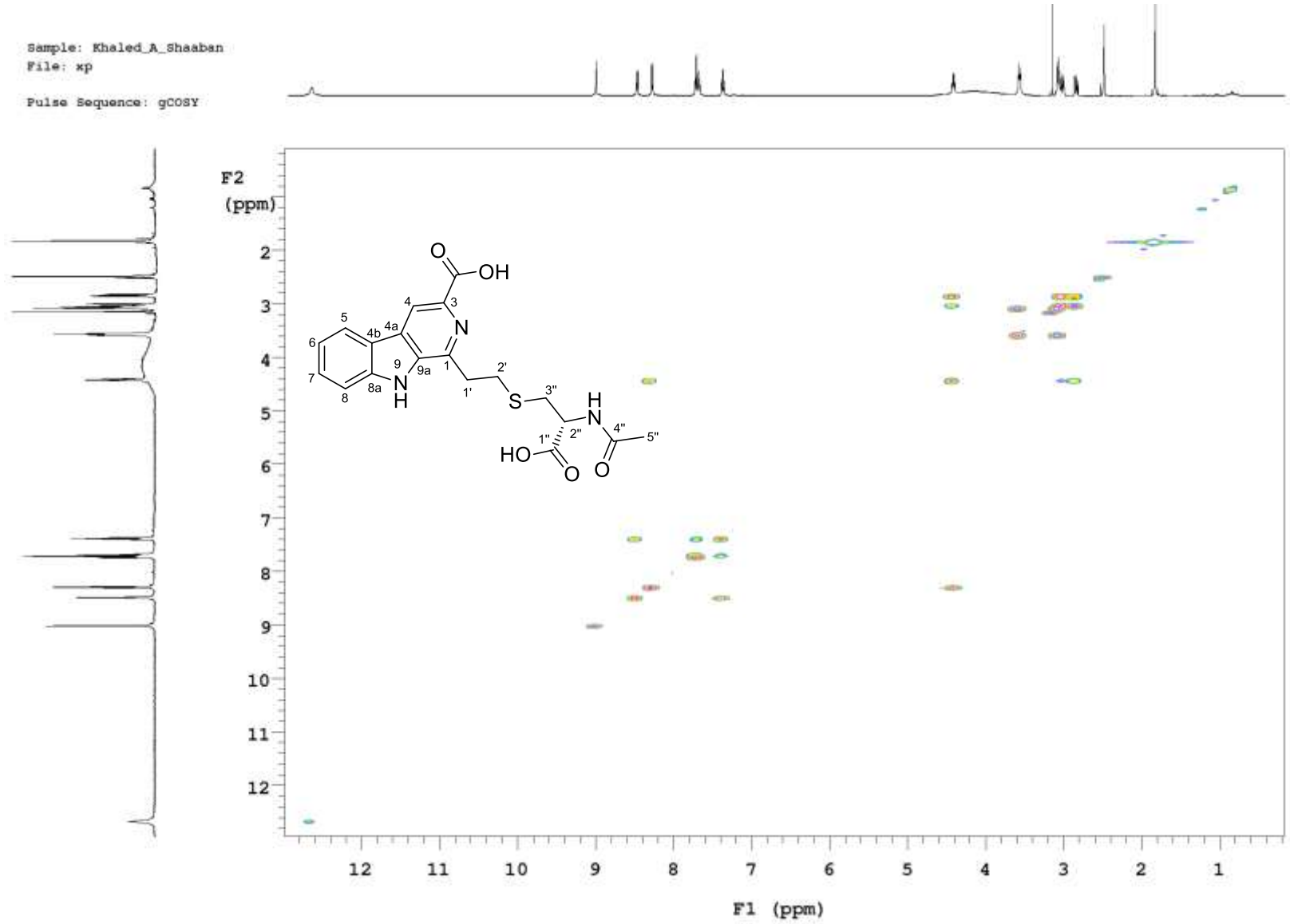

Figure S22: ^{1}H-^{1}H COSY spectrum (DMSO-d_6, 500 MHz) of Kitasetaline (**3**)

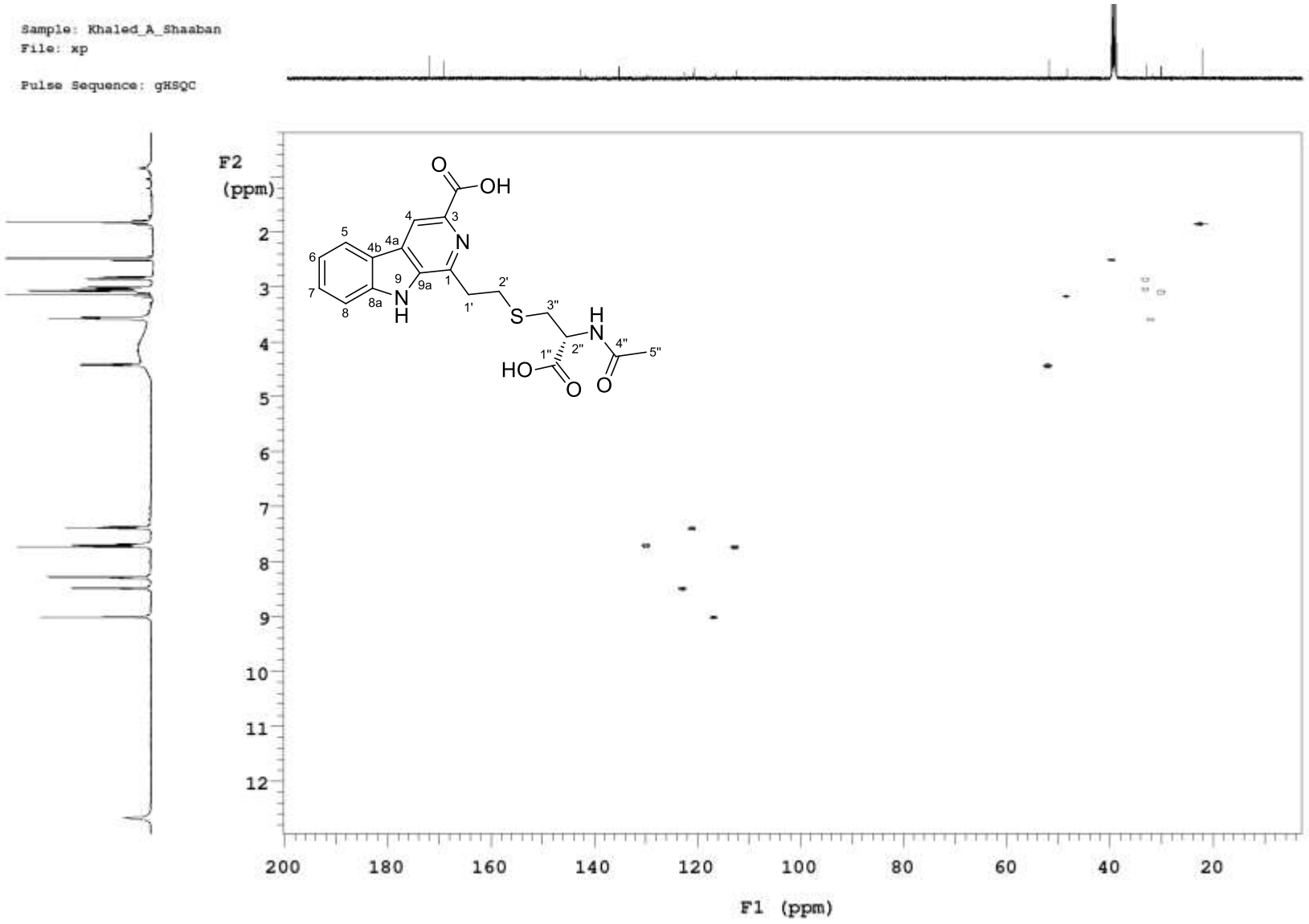

Figure S23: HSQC spectrum (DMSO-d_6, 500 MHz) of Kitasetaline (**3**)

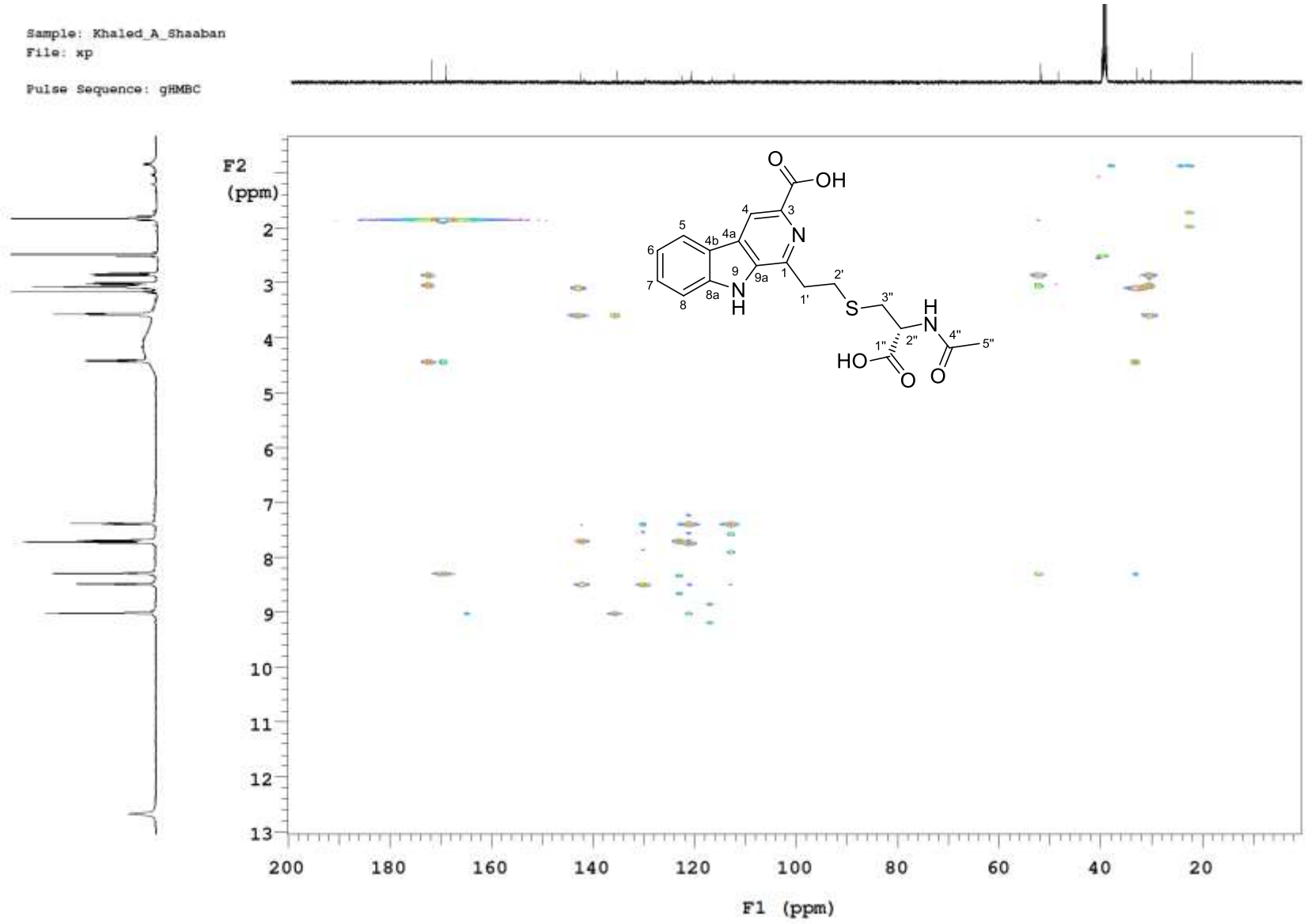

Figure S24: HMBC spectrum (DMSO-d_6, 500 MHz) of Kitasetaline (**3**)

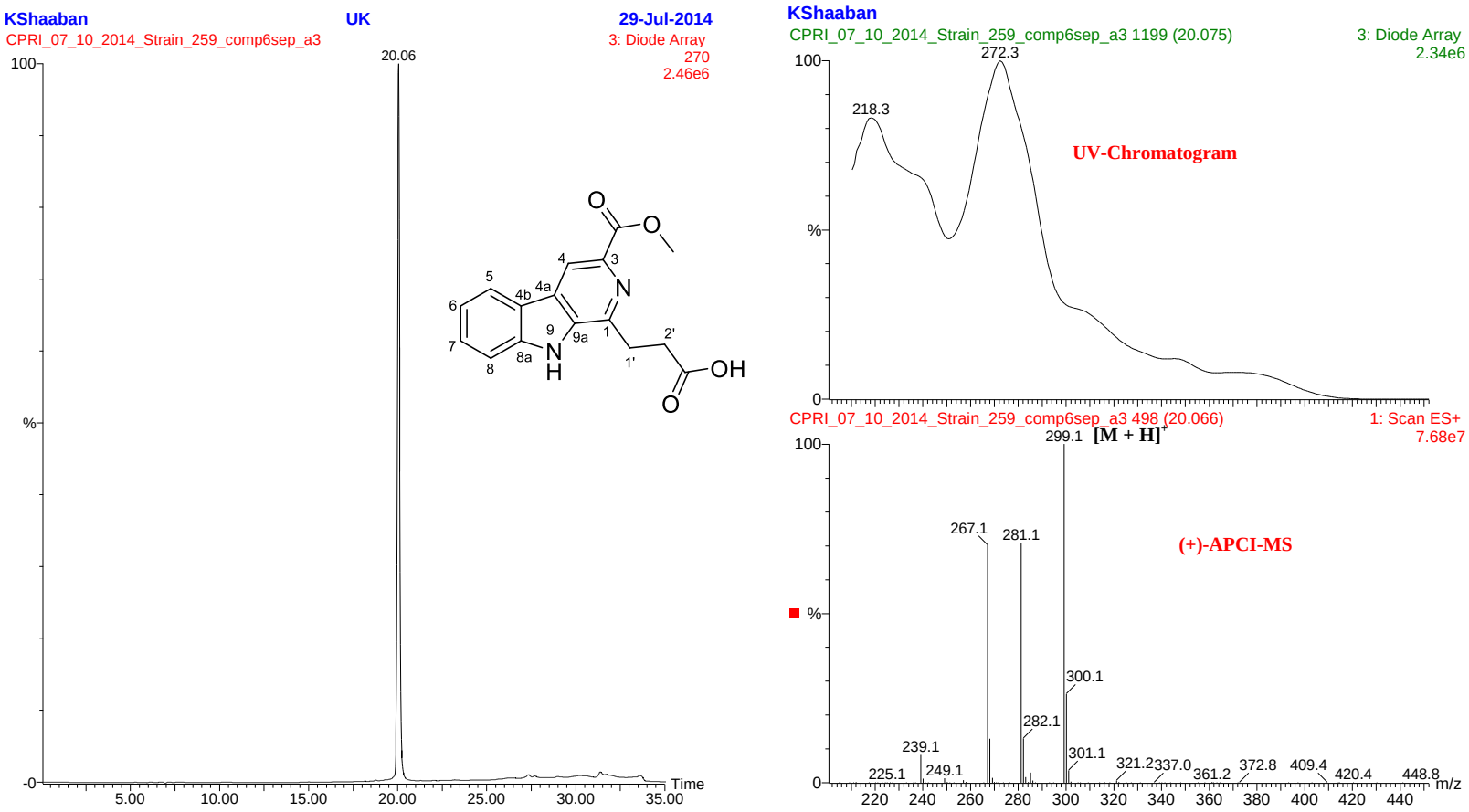

Figure S25: HPLC/UV/APCI-MS analyses of 1-(Propionic acid)-*β*-carboline-3-carboxylic acid methyl ester (**4**). HPLC-conditions: Detection wavelength 270 nm; solvent A: H_2O/0.1% Formic acid; solvent B: acetonitrile; flow rate: 0.5 mL min^{-1}; 0-4 min, 90% A; 4-22 min, 90-0% A (linear gradient); 22-27 min 0% A; 27-35 min 0-90% A (linear gradient).

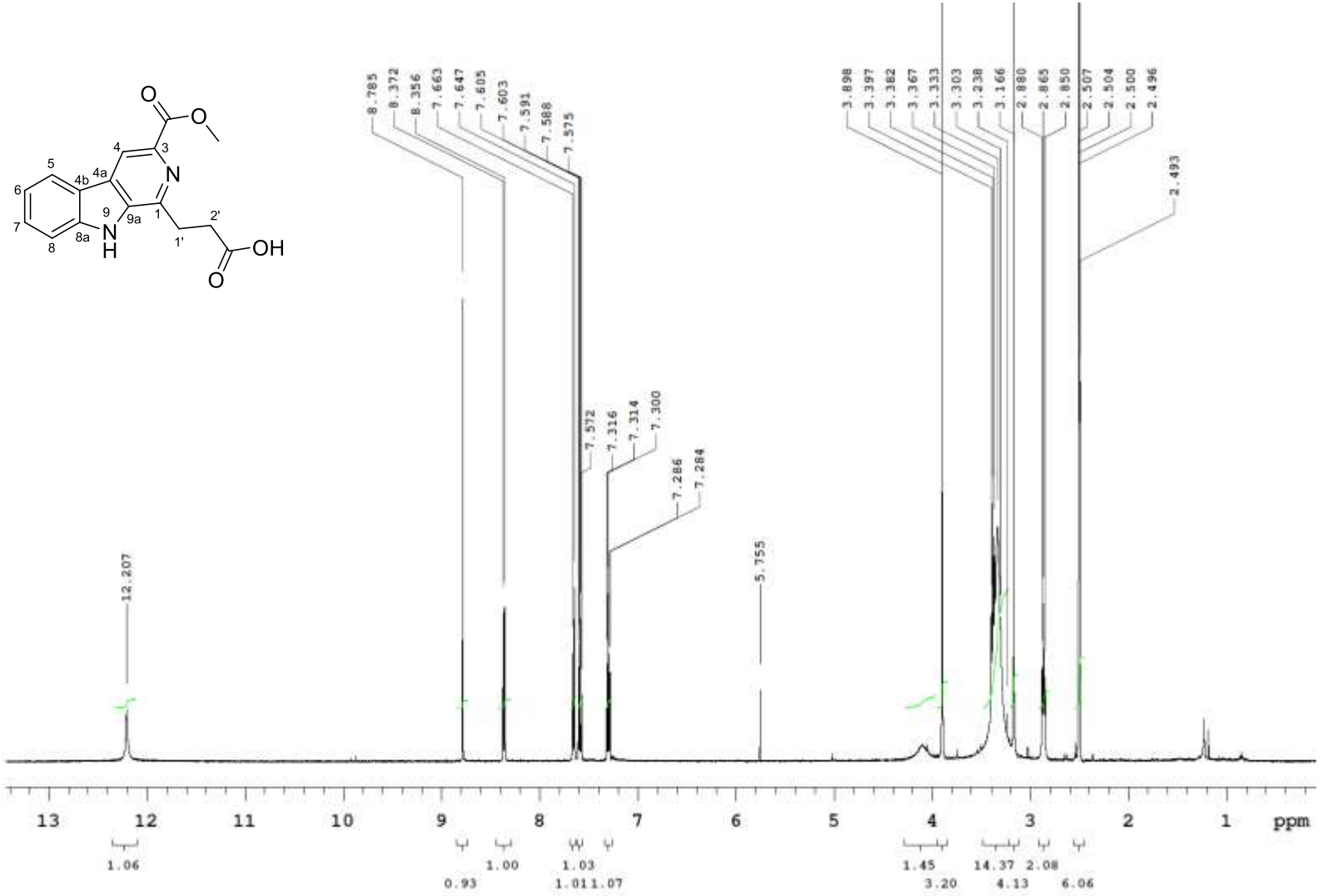

Figure S26: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of 1-(Propionic acid)-β-carboline-3-carboxylic acid methyl ester (**4**)

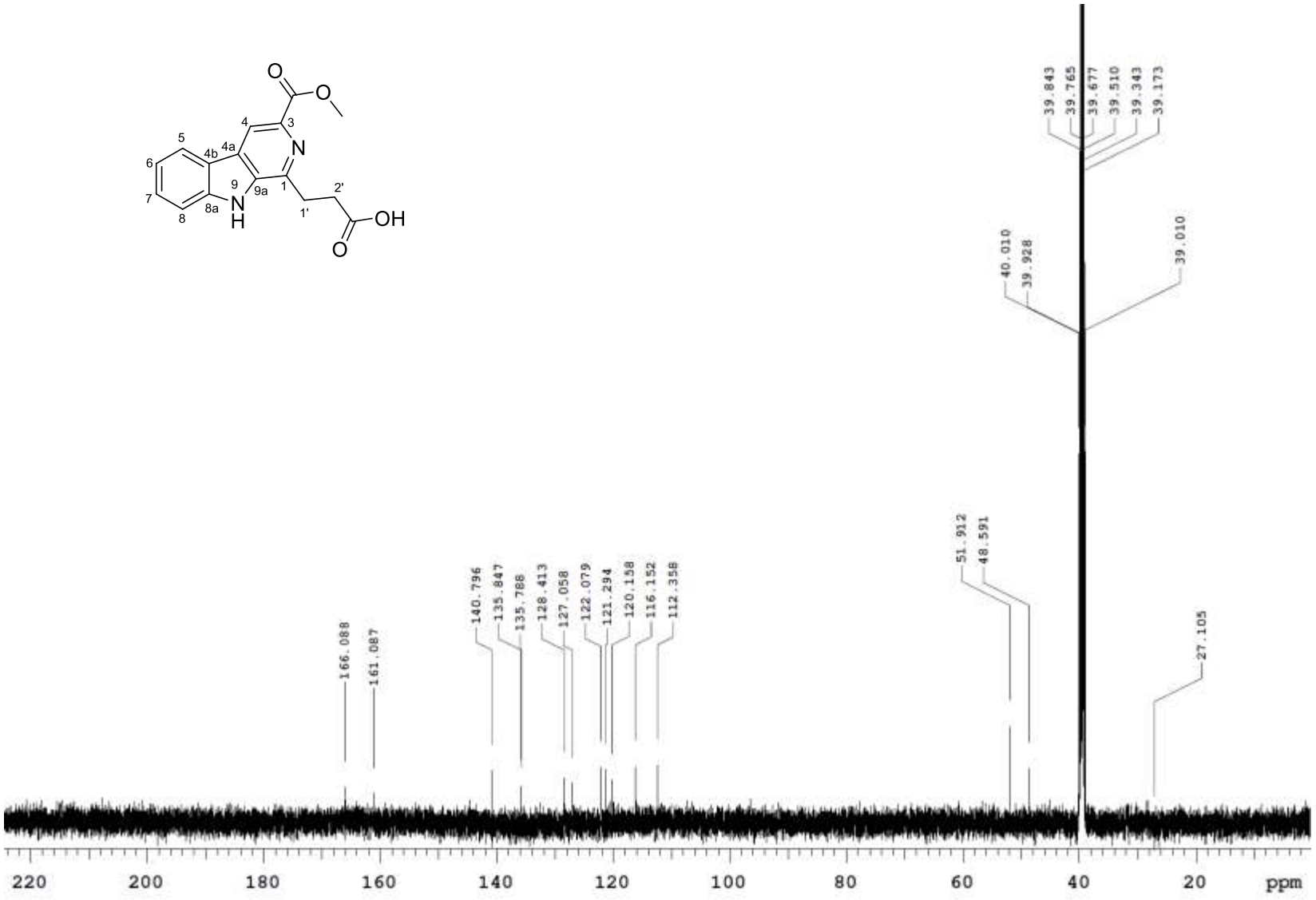

Figure S27: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of 1-(Propionic acid)-β-carboline-3-carboxylic acid methyl ester (**4**)

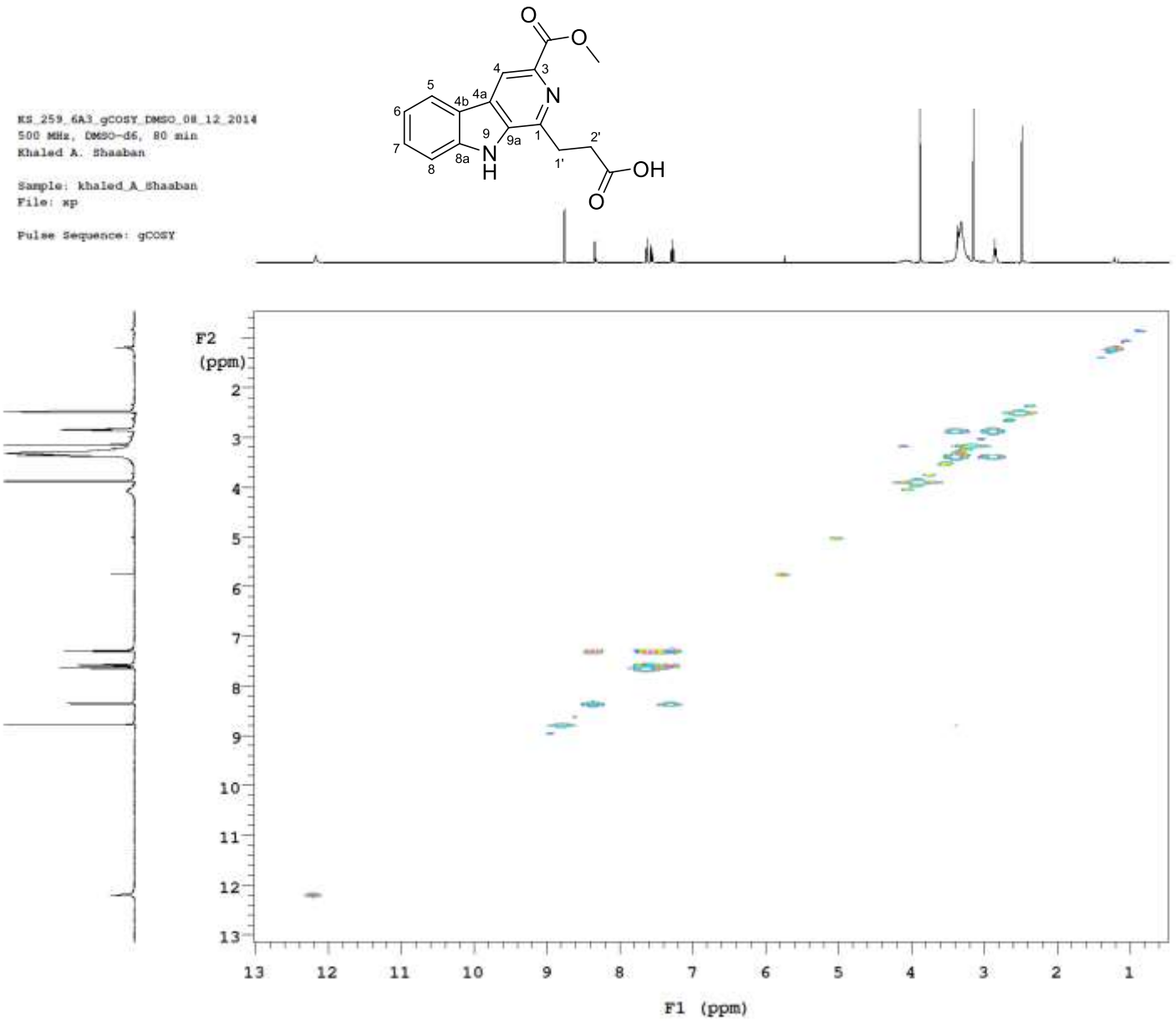

Figure S28: ^{1}H-^{1}H COSY spectrum (DMSO-d_6, 500 MHz) of 1-(Propionic acid)-β-carboline-3-carboxylic acid methyl ester (**4**)

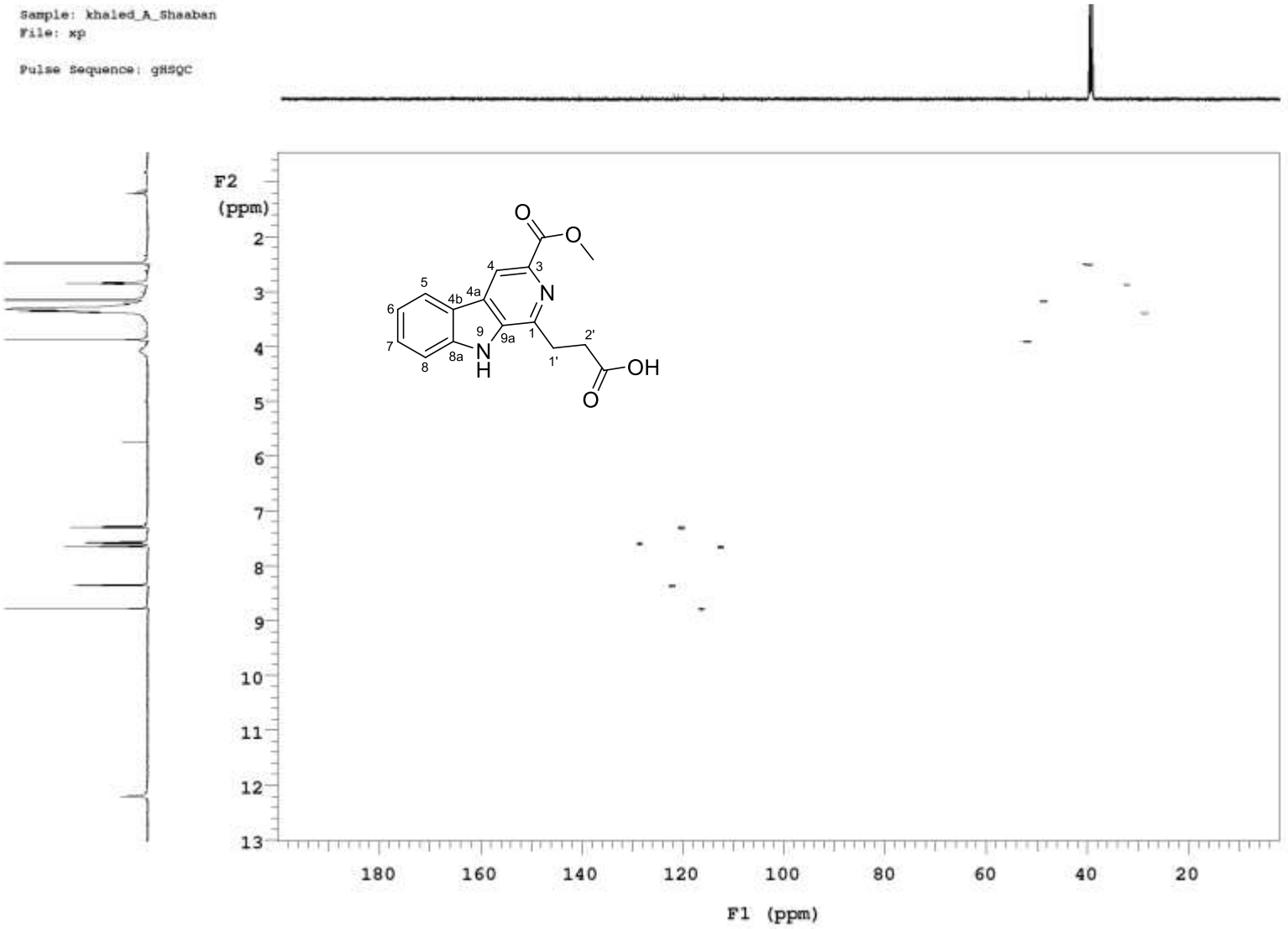

Figure S29: HSQC spectrum (DMSO-d_6, 500 MHz) of 1-(Propionic acid)-β-carboline-3-carboxylic acid methyl ester (**4**)

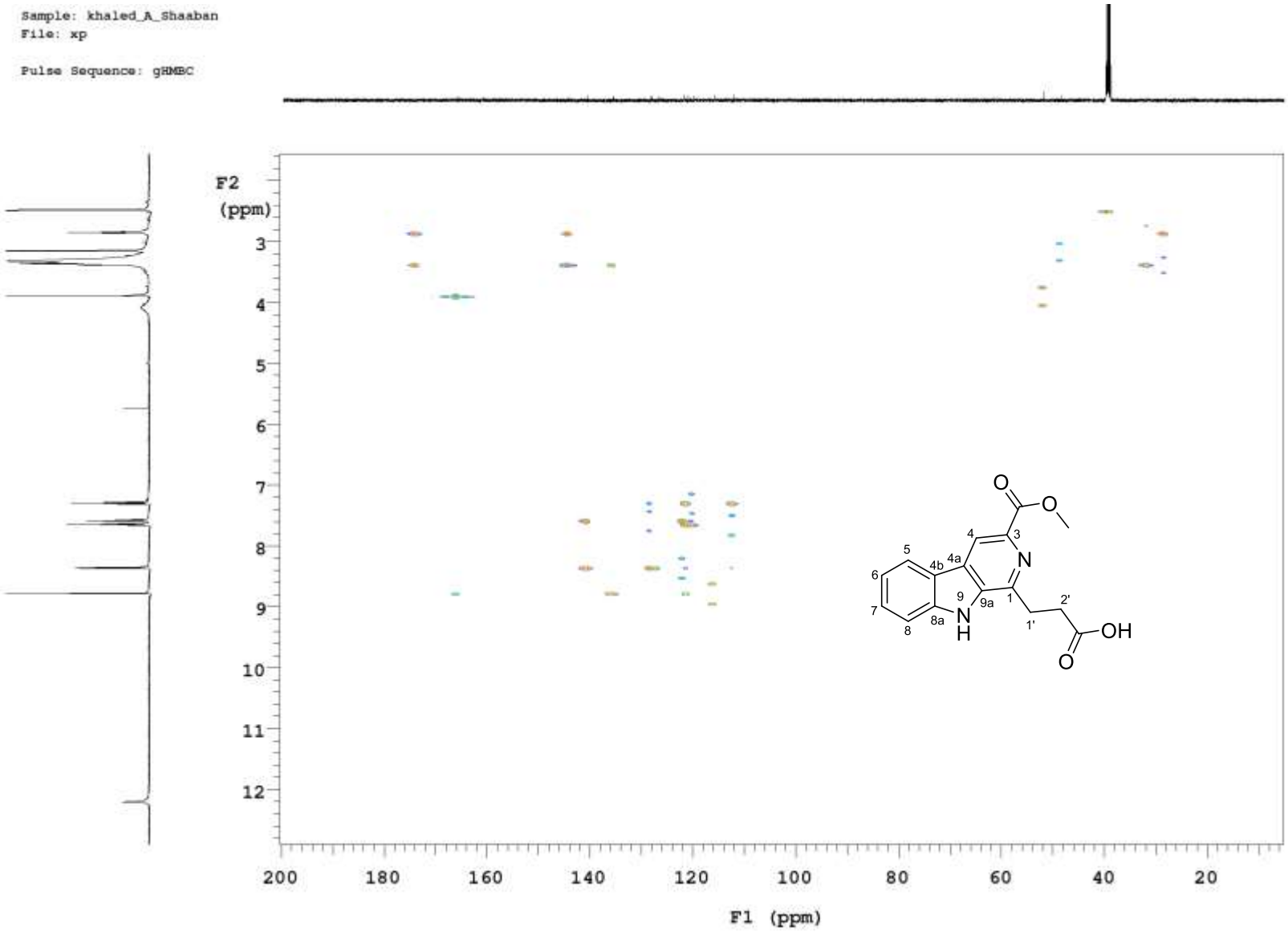

Figure S30: HMBC spectrum (DMSO-d_6, 500 MHz) of 1-(Propionic acid)-β-carboline-3-carboxylic acid methyl ester (**4**)

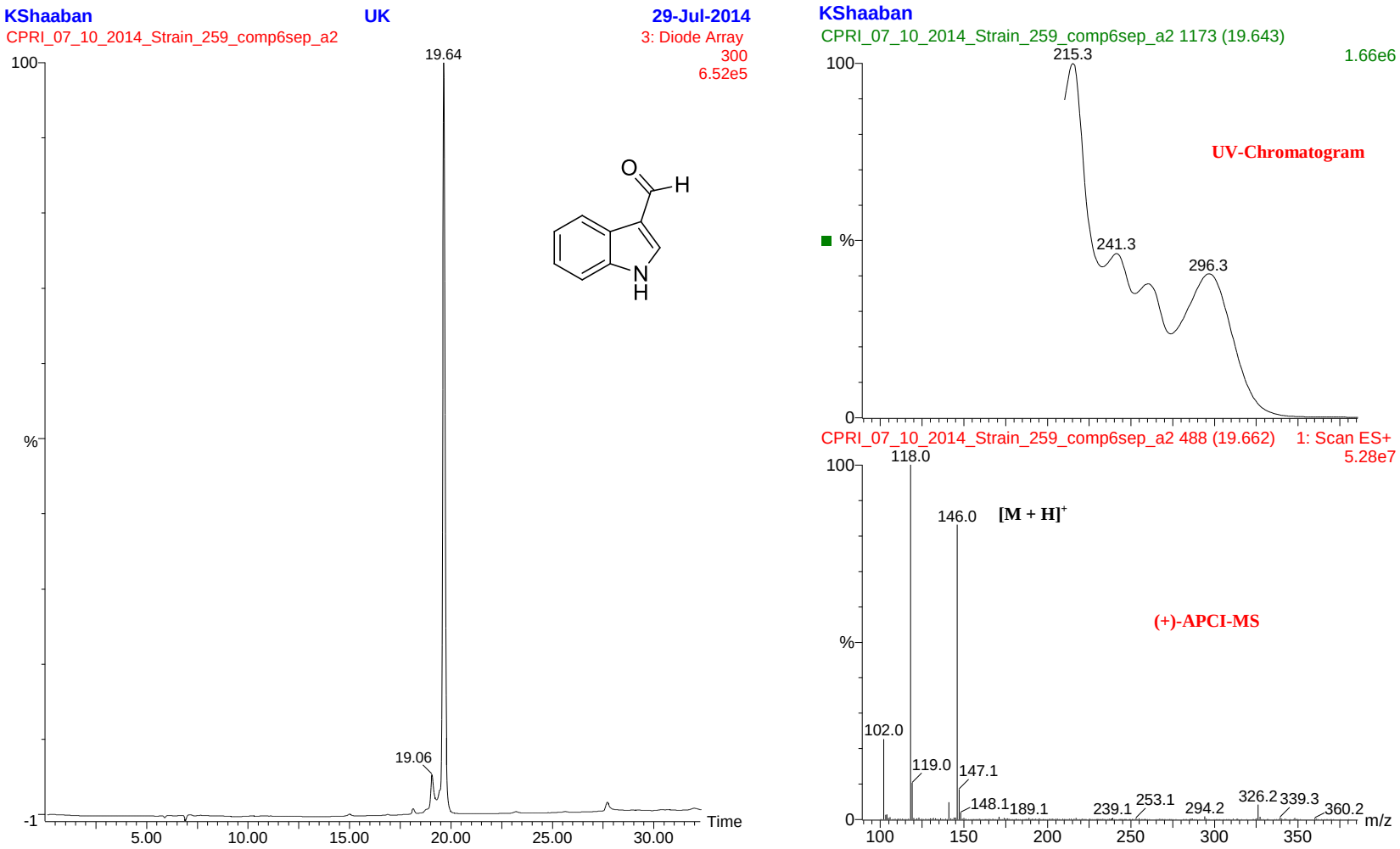

Figure S31: HPLC/UV/APCI-MS analyses of Indole-3-carbaldehyde (**5**). HPLC-conditions: Detection wavelength 270 nm; solvent A: H_2O/0.1% Formic acid; solvent B: acetonitrile; flow rate: 0.5 mL min^{-1}; 0-4 min, 90% A; 4-22 min, 90-0% A (linear gradient); 22-27 min 0% A; 27-35 min 0-90% A (linear gradient).

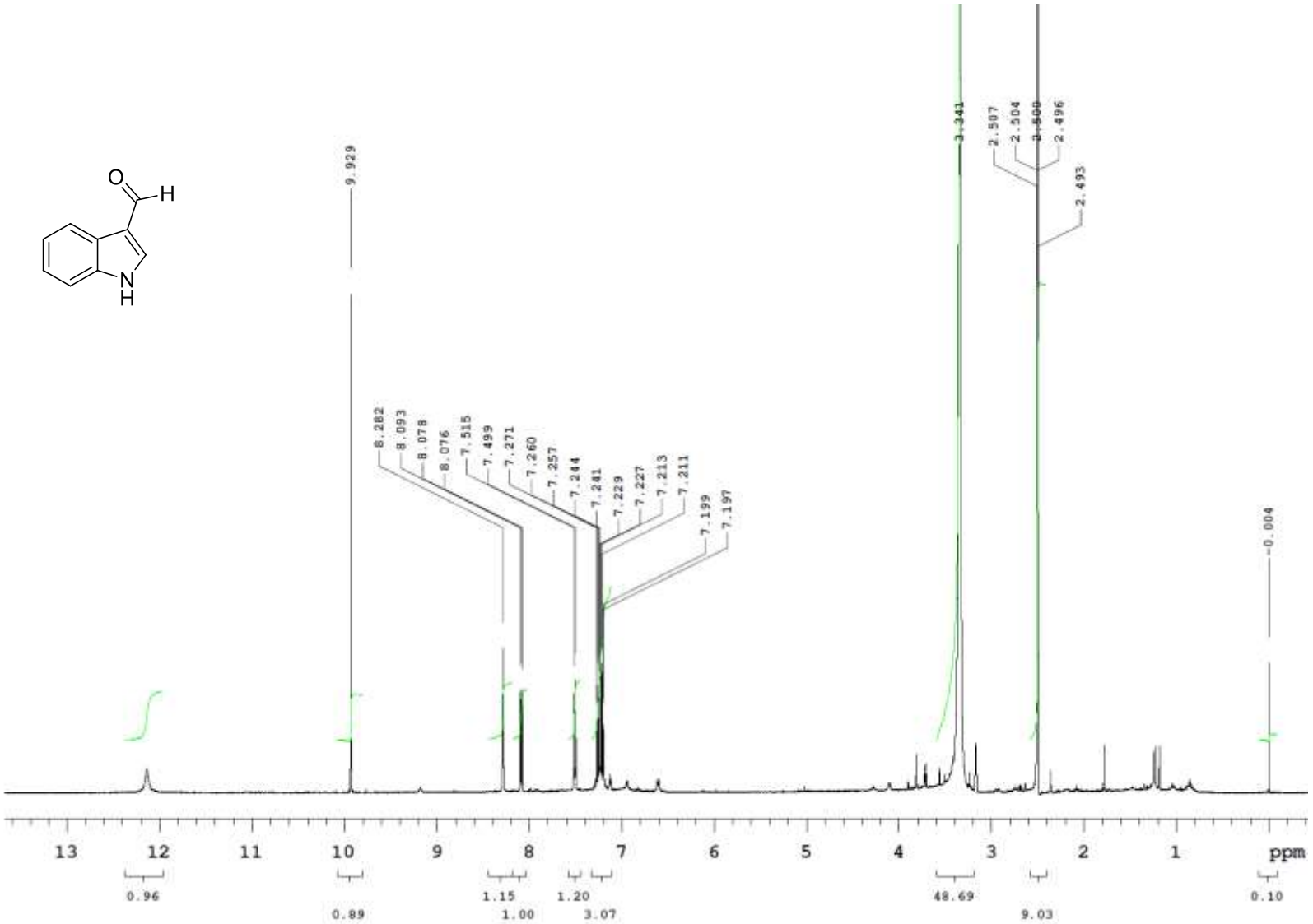

Figure S32: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of Indole-3-carbaldehyde (**5**)

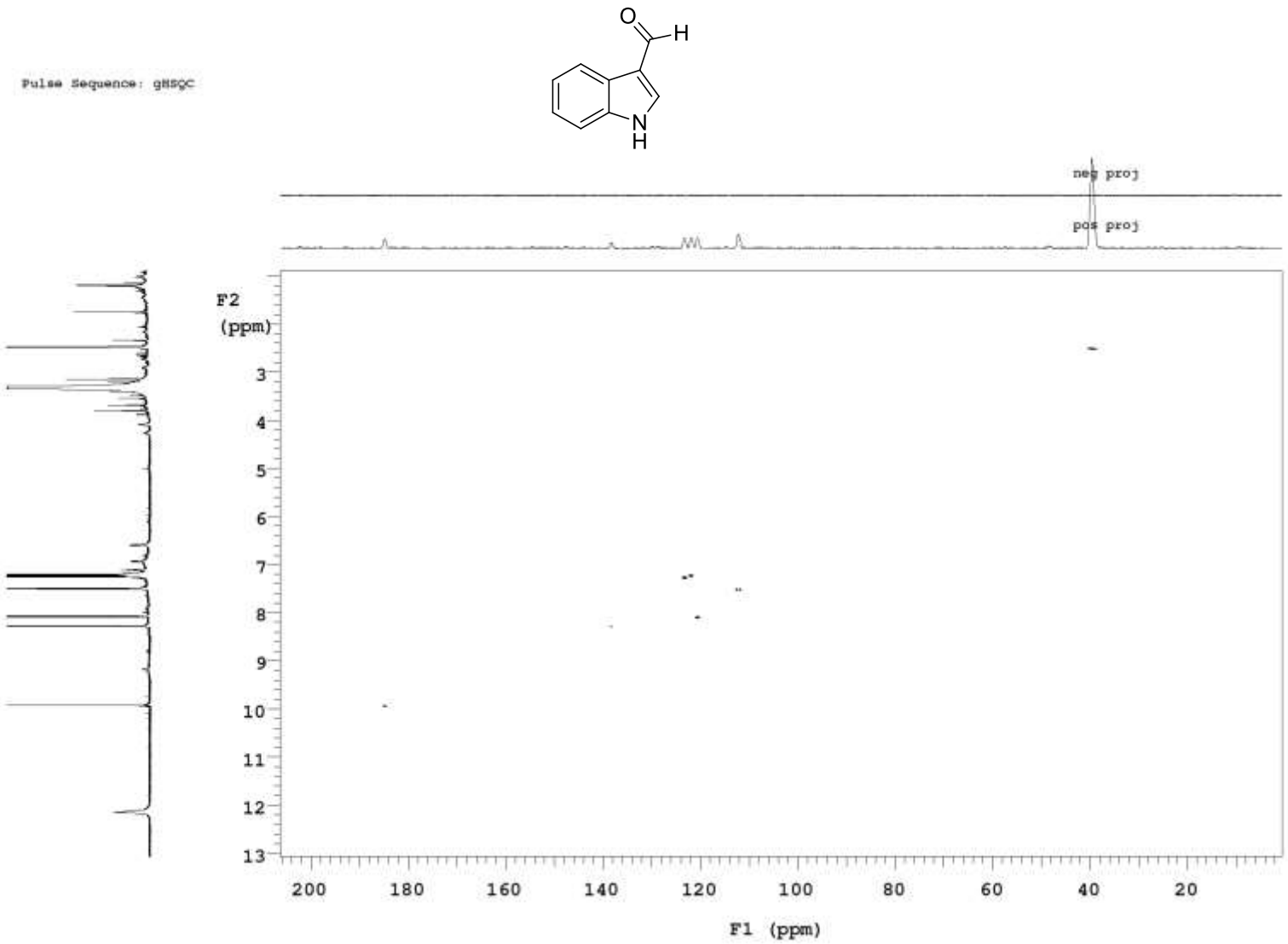

Figure S33: HSQC spectrum (DMSO-d_6, 500 MHz) of Indole-3-carbaldehyde (**5**)

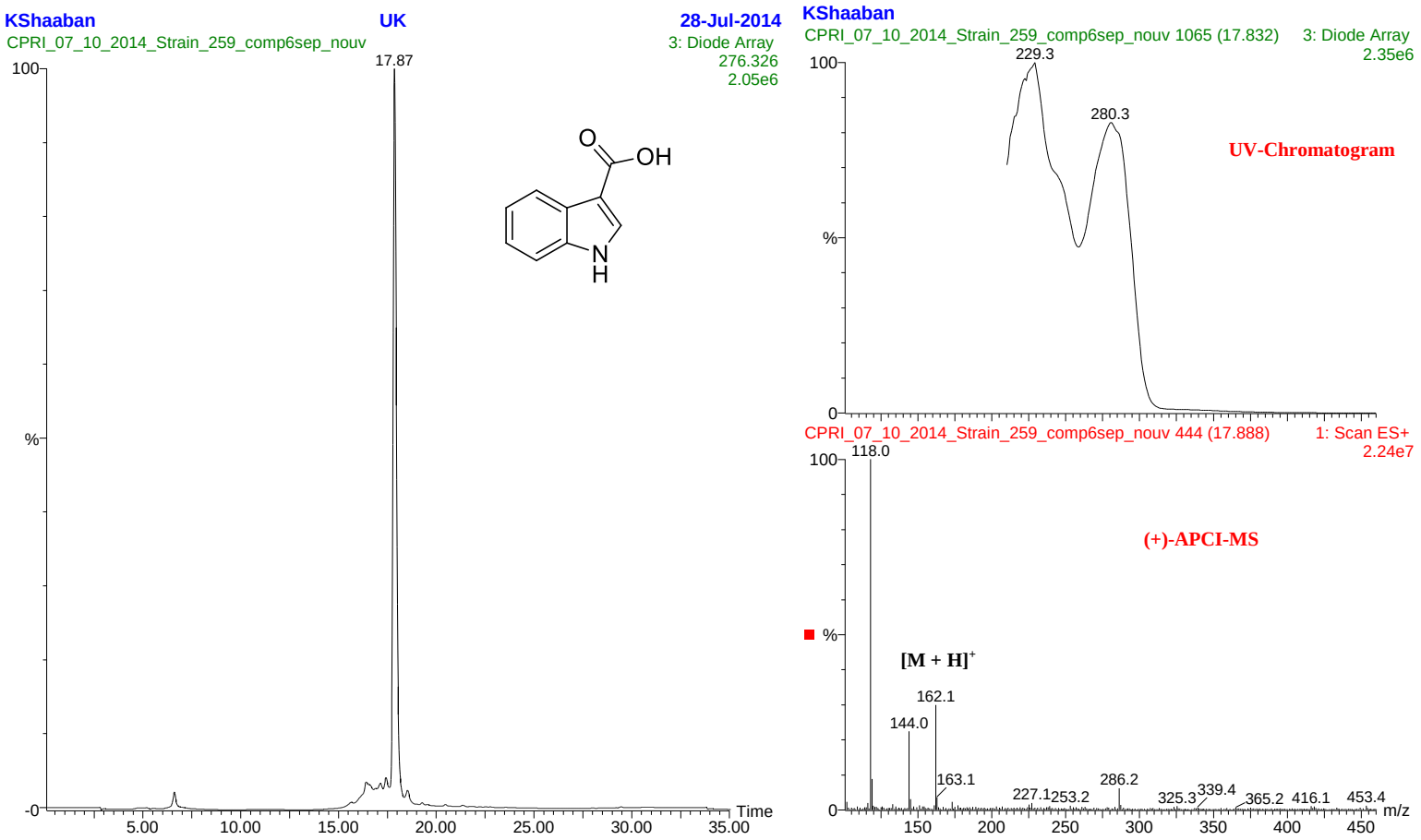

Figure S34: HPLC/UV/APCI-MS analyses of Indole-3-carboxylic acid (**6**). HPLC-conditions: Detection wavelength 270 nm; solvent A: H_2O/0.1% Formic acid; solvent B: acetonitrile; flow rate: 0.5 mL min^{-1}; 0-4 min, 90% A; 4-22 min, 90-0% A (linear gradient); 22-27 min 0% A; 27-35 min 0-90% A (linear gradient).

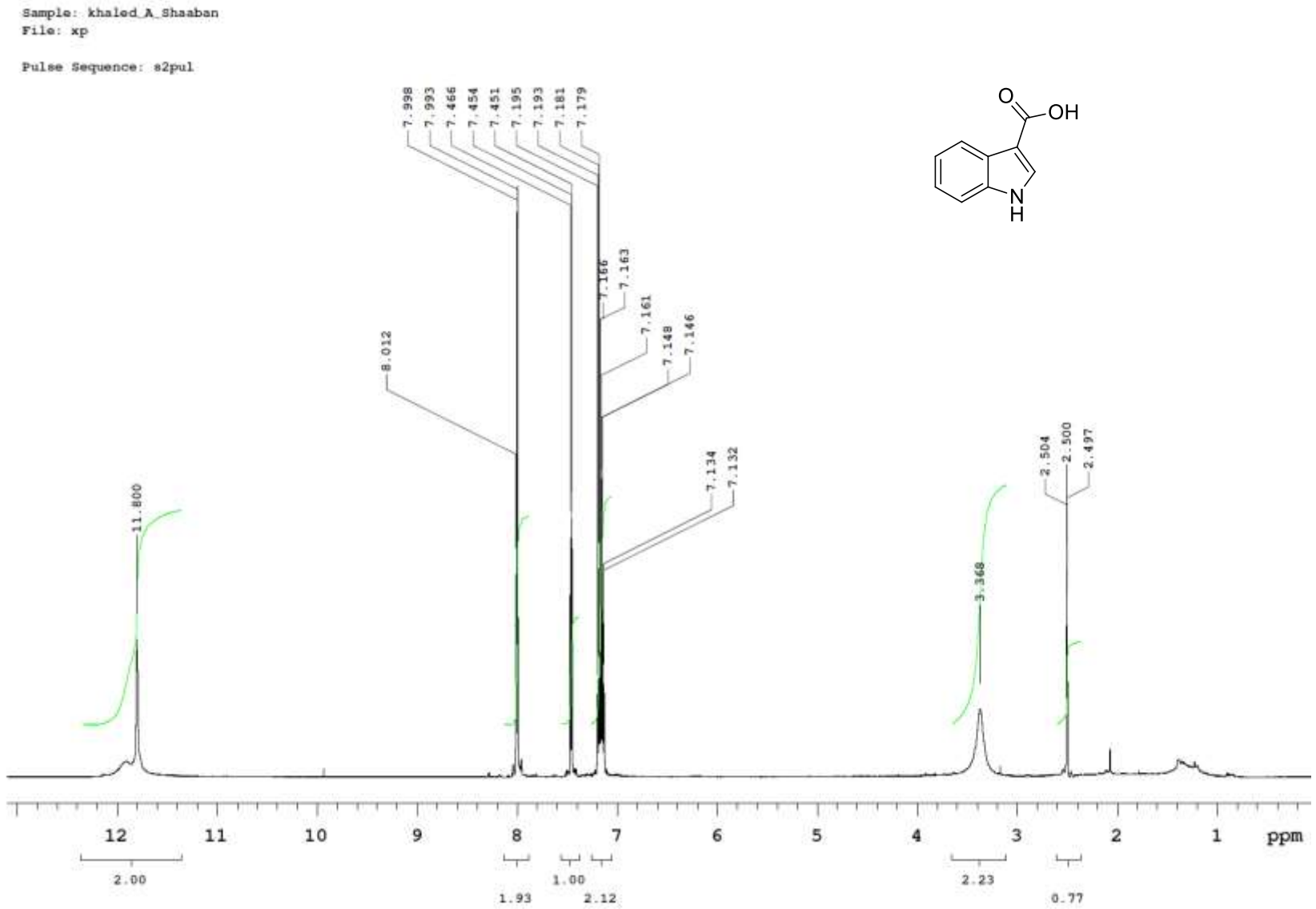

Figure S35: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of Indole-3-carboxylic acid (**6**)

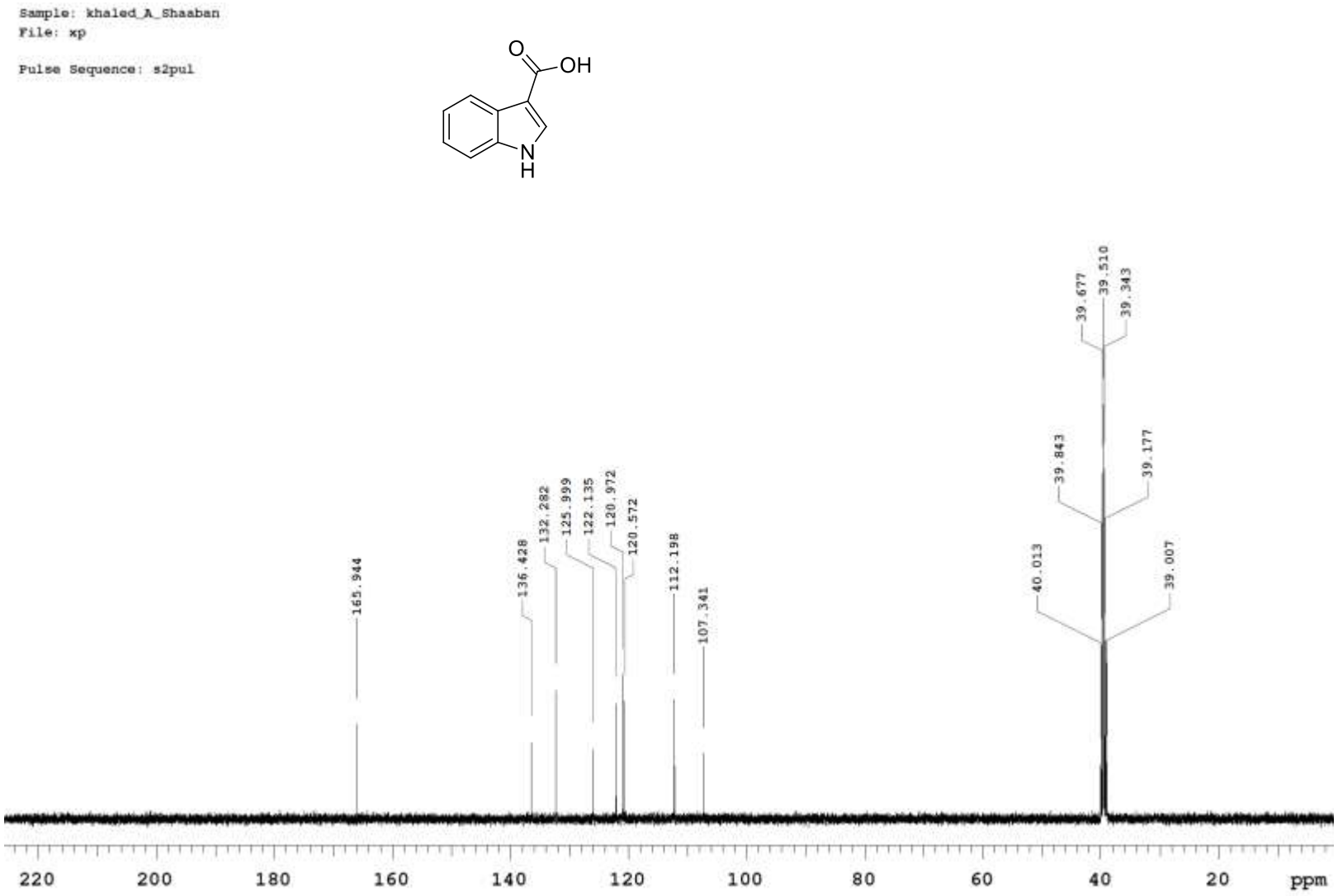

Figure S36: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of Indole-3-carboxylic acid (**6**)

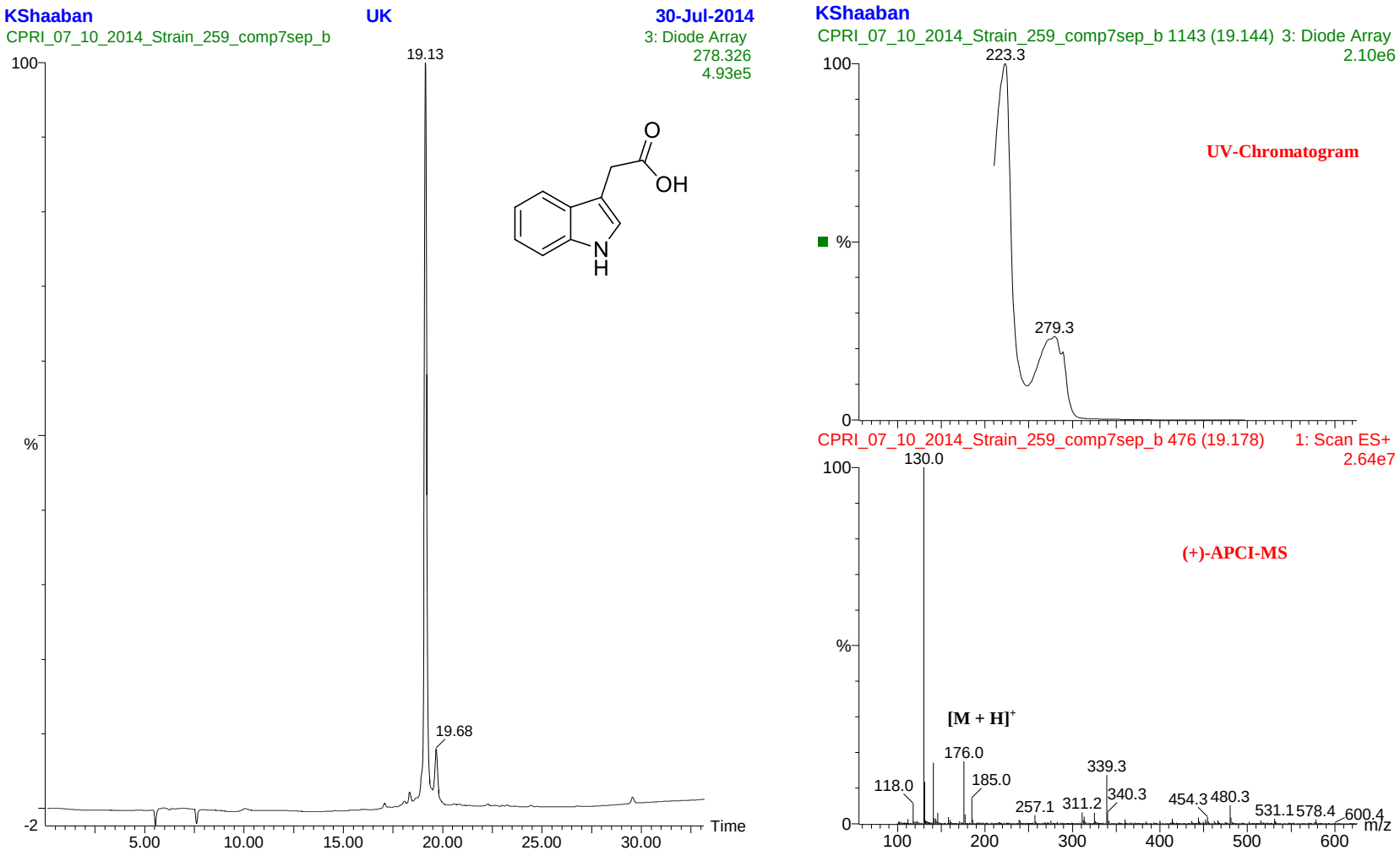

Figure S37: HPLC/UV/APCI-MS analyses of Indole-3-acetic acid (**7**). HPLC-conditions: Detection wavelength 270 nm; solvent A: H_2O/0.1% Formic acid; solvent B: acetonitrile; flow rate: 0.5 mL min^{-1}; 0-4 min, 90% A; 4-22 min, 90-0% A (linear gradient); 22-27 min 0% A; 27-35 min 0-90% A (linear gradient).

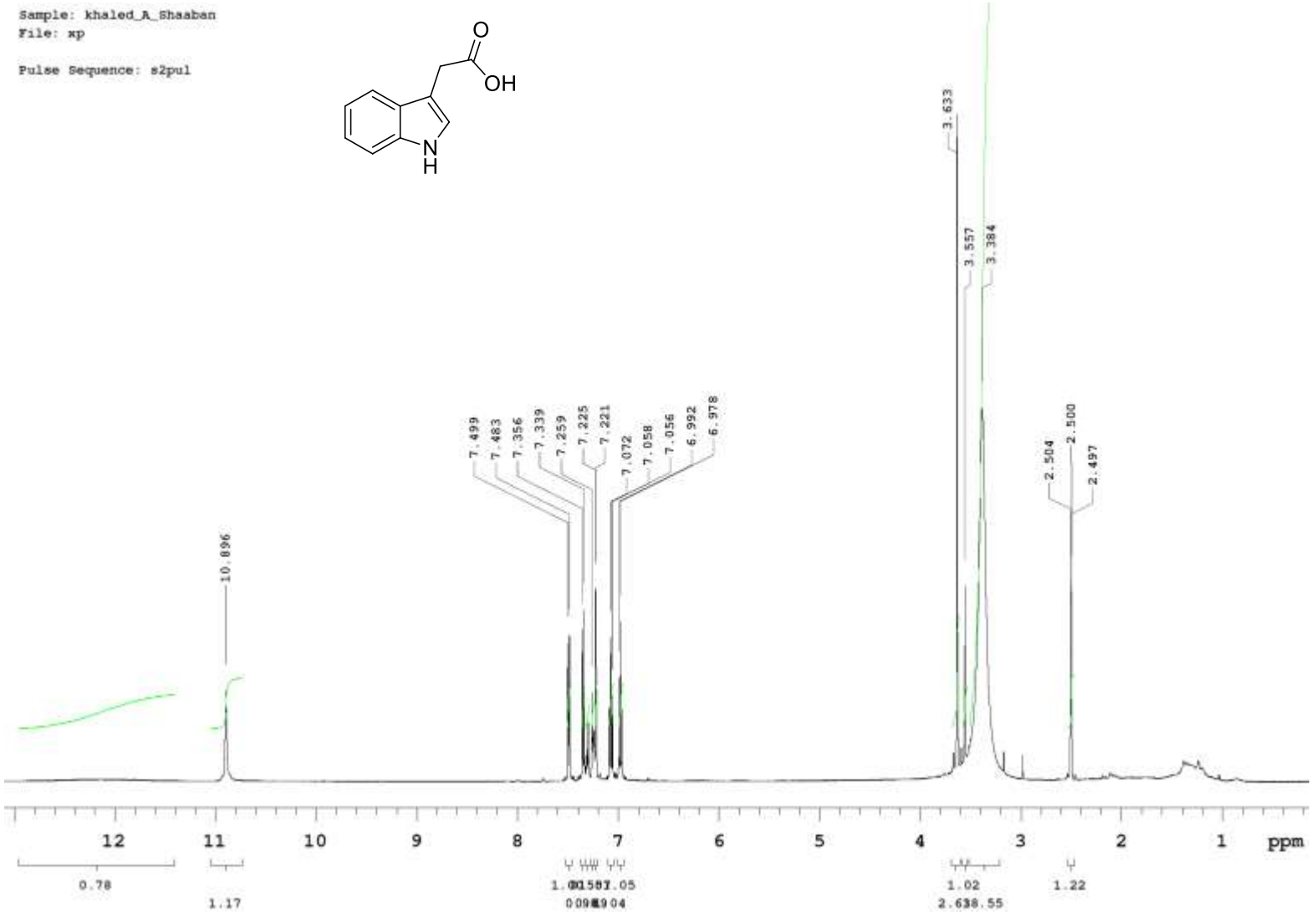

Figure S38: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of Indole-3-acetic acid (**7**)

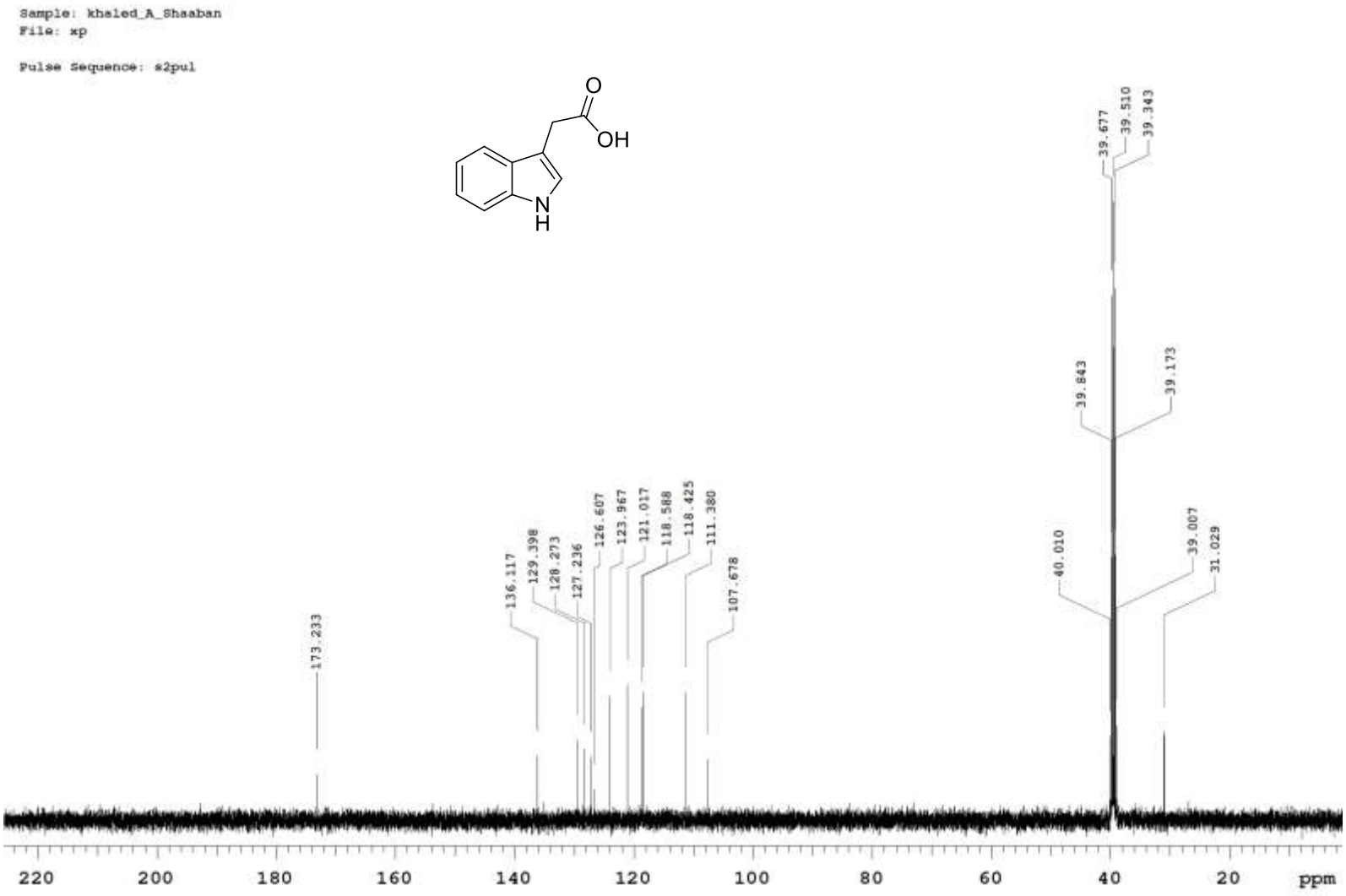

Figure S39: ^{13}C NMR spectrum (DMSO-d_6, 125 MHz) of Indole-3-acetic acid (**7**)

VIII. CAPÍTULO 3 – A ser submetido na revista *International Journal of Systematic and Evolutionary Microbiology*

Multilocus Sequence Analysis of the Genus *Microbispora*

SAVI, D.C. [1]; ALUIZIO, R. [2]; GLIENKE, C. [2]

Affiliations: [1]Department of Pathology, Universidade Federal do Paraná, Av. Coronel Francisco Heráclito dos Santos, 210. CEP: 81531-970, Curitiba, PR, Brazil. [2]Department of Genetics, Universidade Federal do Parana, PO.BOX 19071. CEP: 81531-970, Curitiba, PR, Brazil.

ABSTRACT

The genus *Microbispora* has been considered to be a taxonomically difficult group, wherein 16S rRNA analysis is required to accurately discriminate among phylogenetic relationships of the species, most branches of 16S rRNA-based phylogenetic trees are not reliable. In this study, a Multilocus Sequence Analysis (MLSA) was used to refine the phylogenetic analysis of the genus *Microbispora*. By the time this study started *Microbispora* genus contains 5 species with validly-published names, *M. amethystogenes, M. corallina, M. mesophila, M. siamensis* and *M. rosea* - that containing nine species combined as a single taxa; *M. chromogenes, M. diastatica, M. parva, M. indica, M. karnatakensis, M. rosea, M. aerata, M. thermodiastatica* and *M. thermorosea*. Sequences were obtained for the 16S rRNA, 23S rRNA, *gyr*B and *rpo*B for all type strains plus eleven endophytic isolates from a Brazilian medicinal plant *Vochysia divergens*. A four-gene concatenated sequence of 4486 nt was used to examine the phylogenetic relationships between the species of the genus *Microbispora*. Using the concatenated sequence, most *Microbispora* type strains can be distinguished, with high probability support. The concatenated *gyr*B-*rpo*B tree had good probability support and topologies closest to that of the four concatenated sequence tree. We propose that concatenated *gyr*B-*rpo*B gene sequences be used for examining the phylogenetic relationships within the genus *Microbispora* and indicated that genomic species could be delineated as groups of strains that share >98.0% sequence similarity based on this analysis. The strains isolated from *V. divergens* could not be related to any of the currently described species.

INTRODUCTION

As a rich source of novel bioactive compounds, the genus *Microbispora* has been subjected to intensive isolation and screenings, resulting in various isolates, but a few new species described. In almost all publication regarding the group have classifications going only until genus level. We are particularly interested in endophytes from medicinal plants found in the Pantanal, a unique tropical wetland of Brazil, and we were pioneers in study of endophytic actinomycetes isolated from *Vochysia divergens* a medicinal plant common in the Pantanal. In our search about biodiversity and bioactive compounds, eleven isolates of *Microbispora* genus were obtained from tissues of *V. divergens* (Savi *et al.*, 2014; Savi *et al*., 2015).

The genus *Microbispora* was proposed for actinomycetes that form aerial hyphae bearing longitudinal pairs of spores with *M. rosea* as type species. By the time this work started the genus *Microbispora* contained five species, namely *M. amethystogenes* (Miyadoh *et al.*, 1990; Boondaeng *et al.*, 2009), *M. corallina* (Nakajima *et al.*, 1999), *M. mesophila* (Zhang *et al.*, 1998; Nonomura *et al.*, 1971), *M. siamensis* (Boondaeng *et al.*, 2009) and *M. rosea* (containing nine species combined by DNA–DNA hybridization (DDH) as a single taxa; *M. chromogenes*, *M. diastatica*, *M. indica*, *M. karnatakensis*, *M. parva*, *M. rosea*, which were reduced to as *M. ro*sea subsp. *rosea*, and *M. aerata*, *M. thermodiastatica* and *M. thermorosea*, which were also combined as *M. rosea* subsp. *aerata*) (Miyadoh *et al.*, 1990; Boondaeng *et al.*, 2009).

The large number of isolates and poor species definition caused taxonomic chaos within this genus. The current description of the new species is carried out using 16S rRNA, though with limited phylogenetic success (Nakajima *et al.*, 1999; Boondaeng *et al.*, 2009). Since the 1980s, the advent of molecular techniques has provided a number of genotypic approaches to investigate the taxonomy of *Microbispora*, including rRNA sequence

comparison and DNA–DNA hybridization (Miyadoh *et al.,* 1990; Wang *et al.,* 1996; Zhang *et al.,* 1998). However, due to the respective drawbacks of each method all have their limitations in routine use. DNA–DNA hybridization has been regarded as the gold standard for speciation in prokaryotic taxonomy (Wayne *et al.,* 1987). However DDH offered a simple criterion for species circumscriptions, wider application in different taxonomic groups of organisms has shown problems to differentiate some taxa and indicates mix of others that have distinct phenotypic differences that may merit species recognition (Young *et al.,* 2008). Furthermore, DNA–DNA reassociation studies are intensive and expensive; there is a difficulty to standardization between laboratories, and with increasing numbers of species requiring comparison, it is impractical for all but a few specialist laboratories (Yépez *et al.,* 2014). One of the most useful tools for bacterial identification is 16S rRNA gene-based phylogeny. Although 16S rRNA analysis is required to accurately discriminate among phylogenetic relationships of the *Microbispora* species, most branches of 16S rRNA-based phylogenetic trees are not reliable and do not provide taxonomic resolution between closely related strains (Nakajima *et al.,* 1999; Boondaeng *et al.,* 2009; Savi *et al.,* 2015).

It is important to unravel the taxonomic relationships of *Microbispora* strains at species level to guide the species discrimination and the discovery of potentially novel species for ecological reasons and industrial purposes. MLSA (Multilocus Sequence Analyze) has been successfully applied to phylogenetic analysis of highly diverse bacterial groups, such as the genera *Mycobacterium* (Devulder et al., 2005), *Streptomyces* (Labeda *et al.,* 2014) *Micromonospora* (Carro *et al.,* 2012) and *Kribbella* (Curtis & Meyers, 2012).

In the present investigation, a *Microbispora* MLSA was developed to clarify the taxonomic structure. We sequenced the partial sequences of genes *gyr*B, *rpo*B, 23S rRNA and 16S rRNA for twelve type strains and eleven endophytics strains previous isolated from *Vochysia divergens,* compared the phylogenetic trees derived from the sequence data,

constructed a finer and more robust phylogeny. This study offers a primary multilocus framework for amending the systematics of *Microbispora*, which facilitates our understanding of phylogeny and evolution of this genus.

METHODS

Bacterial strains and culture conditions

Of the 23 strains used in this study (listed in Table 1), eleven were from LabGeM culture collection (Laboratorio de Genetica de Microrganismos, Universidade Federal do Parana, Brazil); six were from NITE culture collection (National Institute of Technology and Evaluation, Japan), and six strains were from JCM culture collection (Japan Collection of Microorganisms, Japan). Strains were cultured in oatmeal extract agar ISP 3 (Shirling & Gottlieb, 1966) at 28, 36 and 50 ºC. The morphologic analyses were performed on ISP 2, ISP 3 and ISP 4 culture media.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from cultures grown on ISP 3 using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to manufacturer's protocol.

The amplification of genes 16S rRNA, 23S rRNA, gyrB and rpoB were realized using 12.5 µl amplification reaction contained 1 µl template DNA (10 ng), 1.25 µl 10X PCR buffer, 0.25 µl each PCR primer (10 mM), 1 µl dNTP mix (10 mM), 0.35 µl MgCl2 (25 mM), 2.5 U Taq DNA and 8 µl sterile MilliQ water. The reaction conditions were initial denaturation at 95 ºC for 5 min, followed by 30 cycles of denaturation at 95 ºC for 30 s, annealing for 30 s at the primer-pair-specific annealing temperature (Table 2) and extension at 72 ºC for 90 s. A final extension was performed at 72 ºC for 10 min. Reaction products were electrophoresed on a 1,5% agarose gel and checked with GelRed® (Life Technologies, USA) under UV light. The product of gene 16S rRNA were ligated in vector pGEM®-T Easy Vector System

(Promega® - USA), cloned in *Escherichia coli* TOP 10 competent cells (SAMBROOK & RUSSEL, 2001), and a second amplification was performed using the primers M13F and M13R. All amplification products were purified using the enzymes Exo1 and FastAP™ (GE Healthcare, USA), and sequenced directly using a Taq DyeDeoxy Terminator Cycle Sequencing Kit and an ABI Prism 3500 automated DNA sequencer (Applied Biosystems).

Data analysis

Summary statistics for the sequences such as G + C content and number of polymorphic sites were computed using DnaSP 5.10.01 (Librado & Rozas, 2009), which was also used to compute synonymous sites and non-synonymous sites with Jukes–Cantor correction statistics, required to calculate the ratio of non-synonymous to synonymous substitutions (dN/dS). Phi was computed for evidence of recombination at 95% confidence interval as previously described and implemented in SplitsTree4 (Huson & Bryant, 2006).

Genes sequences distances, evolutionary model and tree topologies comparisons were estimated using R 3.2.1 (R Core Team, 2015), phangorn (Schliep, 2011), pegas (Paradis, 2010) and APE (Paradis *et al.,* 2004) packages. Two matrixes of phylogenetic distances between all 24 strains were constructed for each single gene and the concatenated sequences, the first one based on similarity percentages and the other one using Kimura's 2-parameters distance (Kimura, 1980). Both were used to compare the single genes discriminatory potential to that of the concatenated sequences.

Phylogenetic analyses were performed throw Bayesian Inference and Maximum Likelihood for each single gene and the concatenated sequences. Bayesian Inference was computed with Mayas 3.2.1 (Ronquist & Huelsenbeck, 2003) using the necessary amount of permutation to reach split frequencies less or equal to 0.01 and discarding the first 25% generated trees. Maximum Likelihood phylogenies were executed at GARLI 2.1 web service (Bazinet *et al.,* 2014) using the best tree search algorithm, it ran until found a 0.99 best tree

probability with the highest possible likelihood score. Both method used the general time reversible (GTR) substitution model, selected based on the Akaike information criterion.

Tree topologies comparisons between single gene and concatenated sequences were evaluated based on two methods, one using Penny & Hendy (1985) rates (PH85) describing the topological distance between two trees as twice the number of internal branches defining different bipartitions of the tips with a single numeric value as output. And other following the branch length score of Kuhner & Felsenstein (1994) to calculate the square root of the sum of the squared differences of the (internal) branch lengths defining similar bipartitions (or splits) in both trees.

Table 1. Strains used in this study and GenBank accession numbers of alleles of loci

Strain	*Origen / code	16S rRNA	23S rRNA	*gyr*B	*rpo*B
Microbispora chromogenes	JCM 3022	KT345224	KT345248	KT362928	KT362952
Microbispora indica	JCM 8971	KT345225	KT345249	KT362929	KT362953
Microbispora karnatakensis	JCM 8972	KT345226	KT345250	KT362930	KT362954
Microbispora parva	JCM 3324	KT345227	KT345251	KT362931	KT362955
Microbispora thermodiastatica	JCM 3110	KT345228	KT345252	KT362932	KT362956
Microbispora thermorosea	JCM 3111	KT345229	KT345253	KT362933	KT362957
Microbispora amethystogenes	NITE 101907	KT345230	KT345254	KT362934	KT362958
Microbispora aerata	NITE 14624	KT345233	KT345257	KT362937	KT362961
Microbispora rosea	NITE 14044	KT345234	KT345258	KT362938	KT362962
Microbispora corallina	NITE 16416	KT345231	KT345255	KT362935	KT362959
Microbispora mesophila	NITE 14179	KT345232	KT345256	KT362936	KT362960
Microbispora siamensis	NITE 104113	KT345235	KT345259	KT362639	KT362963
Microbispora amethystogenes	LGMB 250	KT345236	KT345260	KT362940	KT362964
***Microbispora* sp. 1**	LGMB 251	KT345237	KT345261	KT362941	KT362965
***Microbispora* sp. 3**	LGMB252	KT345238	KT345262	KT362942	KT362966
***Microbispora* sp. 3**	LGMB253	KT345239	KT345263	KT362943	KT362967
Microbispora amethystogenes	LGMB 255	KT345240	KT345264	KT362944	KT362968
Microbispora amethystogenes	LGMB 256	KT345241	KT345265	KT362945	KT362969
Microbispora amethystogenes	LGMB 257	KT345242	KT345266	KT362946	KT362970
***Microbispora* sp. 2**	LGMB 258	KT345243	KT345267	KT362947	KT362971
***Microbispora* sp. 1**	LGMB 259	KT345244	KT345268	KT362948	KT362972
***Microbispora* sp. 3**	LGMB 260	KT345245	KT345269	KT362949	KT362973
***Microbispora* sp. 3**	LGMB 261	KT345246	KT345270	KT392950	KT362974

*JCM: Japan Collection of Microorganisms, NITE: National Institute of Technology and Evaluation, LGMB: Laboratorio de Genética de Microrganismos

Table 2. Primers used for amplification and sequencing

Gene	Primer sequence (5'-3')	Amplicon size (pb)	Annealing temperature	Reference
***gyr*B**	**gyrBPF** GAGGTCGTGCTGACCGTGCTGCACGCGGGCGGCAAGTTCGGC **gyrBPR** GTTGATGTGCTGGCCGTCGACGTCGGCGTCCGCCAT	1100	72	Guo *et al.*,2008
***rpo*B**	**rpoBPF** GAGCGCATGACCACCCAGGACGTCGAGGC **rpoBPR** CCTCGTAGTTGTGACCCTCCCACGGCATGA	970	72	Guo *et al.*,2008
23S rRNA	**ActF** GGTTGGATCCACCTCCTT **ActR** ACCAGTGAGCTATTAGCG	1113	72	Yap *et al.*,1999
16S rRNA	**9F** GAGTTTGATCCTGGCTCAG **1541R** AAGGAGGTGATCCAGCC **785F *** GGATTAGATACCCTGGTAGTC **802R*** TACCAGGGTATCTAATCC	1429	48	Tamura *et al.*,2001

*****primers used only for the sequencing

RESULTS

Sequence attributes

All four gene analyzed were amplified for the type strains *M. chromogenes, M. indica, M. karnatakensis, M. parva, M. thermorosea, M. thermodiastatica, M. amethystogenes, M. aerata, M. rosea, M. corallina, M. mesophila, M. siamensis* and endophytic isolates LGMB250, LGMB251, LGMB252, LGMB253, LGMB255, LGMB256, LGMB257, LGMB258, LGMB259, LGMB260a and LGMB261a. The GenBank accession numbers of the sequences are listed in Table 1. The features of each gene locus are displayed in Table 3. Individual gene sequences contained 1439 nt (16S rRNA), 1031 nt (23S rRNA), 1128 nt (*gyr*B) and 888 nt (*rpo*B). The average G + C ranged from 57,1 to 68,7%. The range of similarity varied for each of the loci from 85,72 to 100% for *gyr*B; 86,31 to 100% for *rpo*B; 91,2 to 100% for 23S rRNA and from 87,59 to 100% for 16S rRNA, evolutionary distance of each loci was from 0,00 to 0,226 for *gyr*B, 0,00 to 0,150 for *rpo*B, 0 to 0,141 for the 23S rRNA and 0 to 0,077 for 16S rRNA (Table 3, Supplementary Information Table S1-

S12). The dN/dS ratio is used to estimate the degree of selection operating on each locus, for *gyr*B gene the dN/dS value was less than 1, indicating that this gene is not subjected to selection pressure for amino acid changes – it is subject to purifying selection, data confirmed by Phi test, that did not show significant events of recombination, value of 0,183. However, the Phi test suggested that the gene *rpo*B had significant high recombination events (0,597), this data is accorded with the dN/dS value of 4.494, suggesting that selection has caused some amino-acid substitutions.

Table 3. Properties of loci

Locus	Allele length (pb)	No. of polymorphic sites	% polymorphic sites	Nucleotide diversity	Mean G + C content (mol%)	Distance (K2P) Min-max: mean*	dN/dS	Phi
***gyr*B**	1128	223	19,76	0.065	66,7	0,00-0,226: 0,070	0,168	0,183
***rpo*B**	888	228	25,67	0.071	68,5	0,00-0,150: 0,075	4,494	0,597
23S rRNA	1031	179	17,36	0.044	57,1	0,00-0,141: 0,046	-	-
16S rRNA	1439	151	10,49	0.026	58,3	0,00-0,077: 0,027	-	-
Concatenated	4486	781	17,40	0.056	62,6	0,00-0,129: 0,051	-	-

*Pairwise distance calculated by using the K2P substitution model.

16S rRNA gene tree

The alignment of 16S rRNA gene sequences produced 1439 nt including gaps. Of these 151 sites were variable. The range of genetic diversity varied from 0,35 to 9,66%, and the overall mean distance from 0 to 0,077 (mean 0,027). Bayesian inference tree showed *M. mesophila* as a single branch and the others strains grouped by a low probability support, low nucleotide sequence diversity and high DNA similarity (Figure 1; Supplementary Information, Table S6 and S11). Analysis of *Microbispora* phylogeny based on 16S rRNA gene sequences shows no phylogenetic support, low information rate and incongruence between the ecological origin of *Microbispora* taxa and their evolution. Consequently it was

impossible to discriminate species on the basis of their respective 16S rRNA, this led us to additional genes.

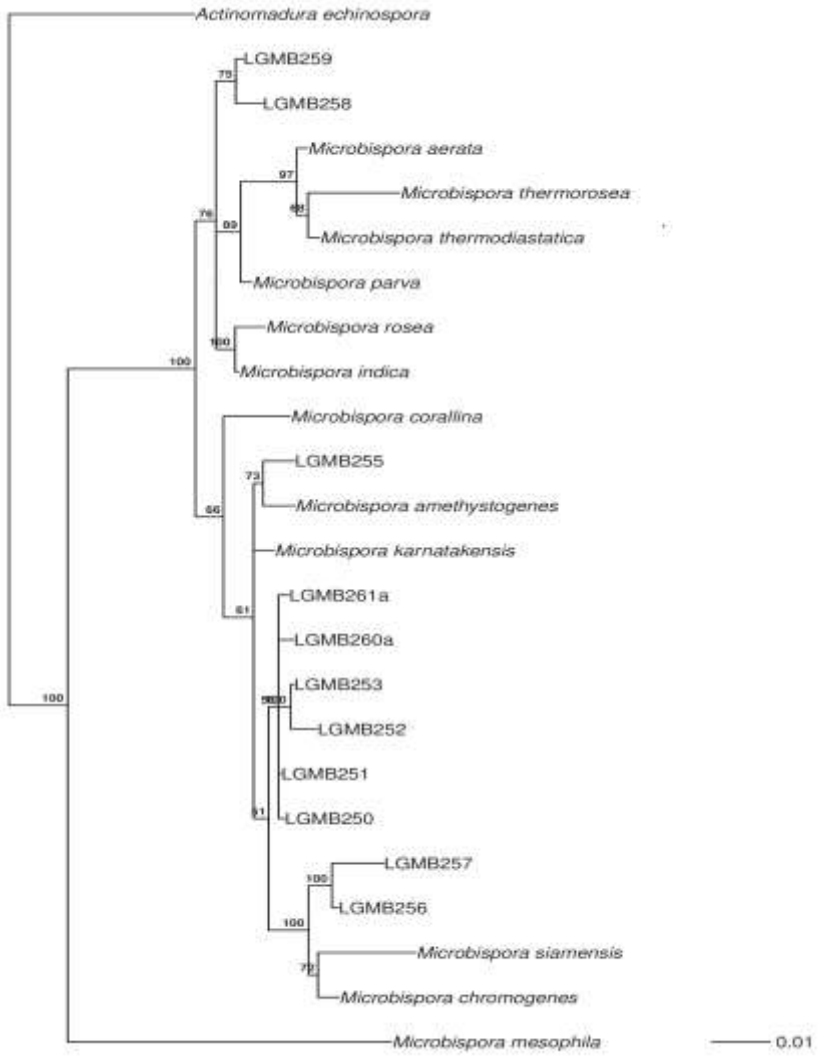

Figure 1. Bayesian phylogeny tree based on 16S rRNA gene sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.01 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

MLSA Data Analysis

The concatenate alignment of four loci counting 4486 nt including gaps, mean G + C content of 62,6%; and 781 variable sites. Nucleotide sequence diversity was found to be

extensive at all 3 loci, in contrast with the almost full-length 16S rRNA gene sequence (Table 3).

Phylogenetic tree predict of four genes concatenate nucleotide sequences showed a better and robust resolution than the 16S rRNA gene (Figure 2). Most of strains formed distinct clade in the MLSA phylogeny, with evolutionary distances ranging from 0,001 to 0,129 (mean 0,051). In the tree based on Multilocus sequences, 18 of 20 nodes were supported by more than 80% of probability, in contrast, the phylogenetic tree based on the 16S rRNA gene sequence had only 8 branch supported by 80% or more of posterior probability, and these, just one branch was not congruent with the MLSA tree. In fact, the concatenation showed an increased in deep-node support values. Therein *M. mesophilla* and *M. corallina* as a single branches, *M. chromogenes* and *M. parva* clustered with posteriori probability of 100%. One cluster with three clades: the first clade consist of *M. indica* and *M. rosea,* had 100% of support, 97,97% similarity and 0,008 of genetic divergence*;* the second with *M. thermodiastatica, M. aerata* and *M. thermorosea* had 94,14 to 99,06% similarity, 0,009 to 0,026 genetic divergence and 100% probability support; and the third *M. karnatakensis* and *M. siamensis*, supported by 100% probability, 98,73% similarity and 0,12 of evolutionary distance. The second cluster was formed by other 3 clades. The first clade formed by strains LGMB255, LGMB256, LGMB257, LGMB250 and the type strain *M. amethystogenes,* with 98,79 to 99,73% similarity and 0,002 to 0,012 evolutionary distance. We address the name *M. amethystogenes* to these four endophytic strains. The others two clades were formed by seven endophytic isolates supported by 100% probability value; LGMB251, LGMB258 and LGMB259 (97,64 to 98,49% of similarity and 0.022 to 0,05 of evolutionary distance) and other compact clade with LGMB252, LGMB253, LGMB260a and LGMB261a supported by 100% probability, with 99,19 to 99,95% of similarity and 0,000 to 0,008 divergence. The MLSA showed evidence that these seven

endophytic isolates might be new species. Strains were well separated on the concatenated phylogenetic analyses, except *M. rosea* and *M. indica*; and *M. aerata* and *M. thermodiastatica* which are high related and share high levels of genetic similarity. Data about nucleotide similarity and evolutionary distance are on Supplementary Information, Tables S1 and S7.

Therefore, we reanalyzed data using various combinations to produce three and two-locus trees (Support Information, Figures S1-S8), most combinations included the 16S rRNA locus because of its predominance in sequence-based identification schemes. The trees were examined to determine whether they yielded the same species clusters and strains placement as the four-locus tree. The phylogenic tree based on *gyr*B-*rpo*B sequences yields the highest correlations with MLSA and showed highest probability support than MLSA tree -19 of 20 nodes had more than 95% probability support - the highest evolutionary distances and lowest nucleotide similarity (Figure 3). The phylogenetic trees using sequences of 23S-*gyr*B-*rpo*B, 16S-23S-*gyr*B, 23S-*gyr*B, 16S-*gyr*B, 23S-*rpo*B showed the same topology, with lowers probability support values, and the 16S-23S-*rpo*B, 16S-*rpo*B and 16S-23S had minor incongruences (Support Information, Figures S1-S8).

Phylogenetic analyses were also constructed for each gene (Supplementary information, Figures S9 e S11). All single-gene trees showed higher resolution than the 16S rRNA tree. Indeed the phylogenic analyses of gene *gyr*B showed the best resolution, contains phylogenetic information to reliably discriminate all species in the tree, but had minor incongruences compared with MLSA and the *gyr*B-*rpo*B trees (e.g. the strains LGMB258 was not clustered on the same clade of LGMB251 and LGMB259) and lower probability support values (Supplementary Information, Figure S11). 23S rRNA phylogenic tree showed almost the same topology but short branches and lower probability support (Support

Information, Figure S9). Consequently, attempts to identify individual strains on the basis of individual loci were unsuccessful.

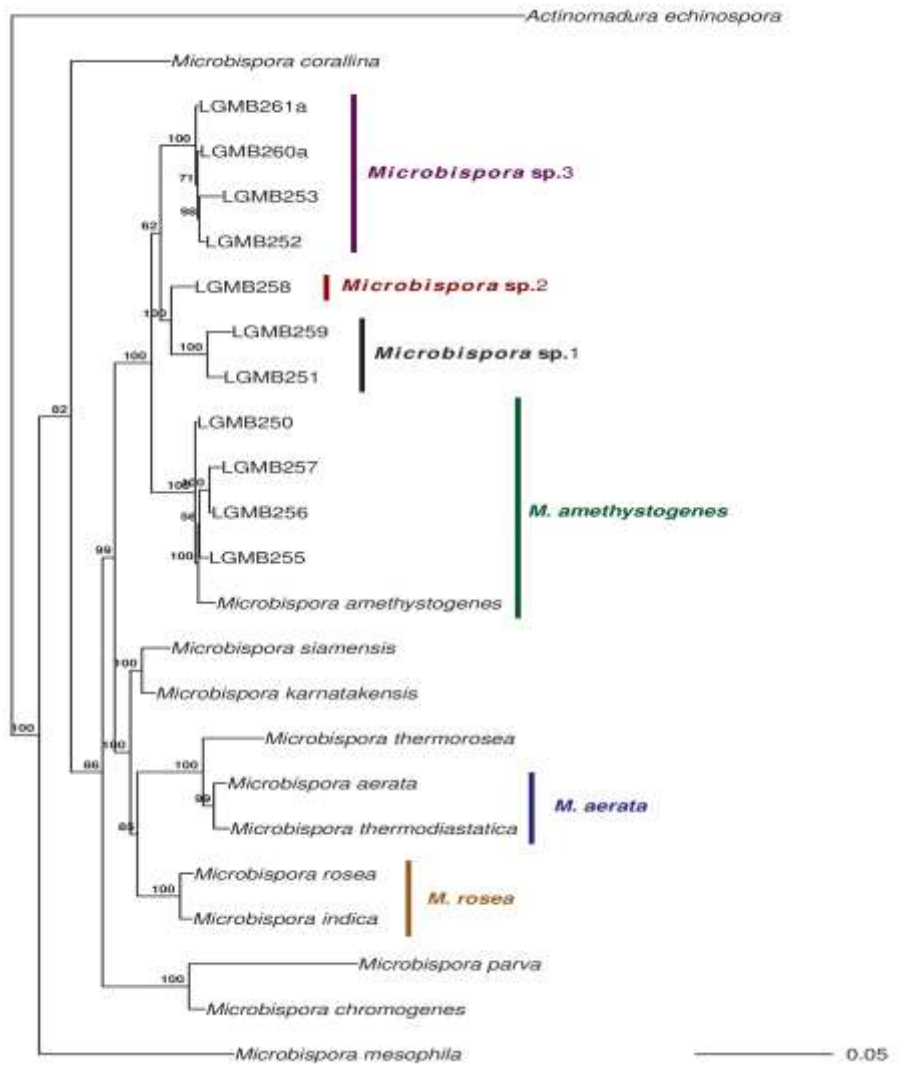

Figure 2. Bayesian phylogeny tree based on 16S rRNA, 23S rRNA, *gyr*B and *rpo*B gene concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.05 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

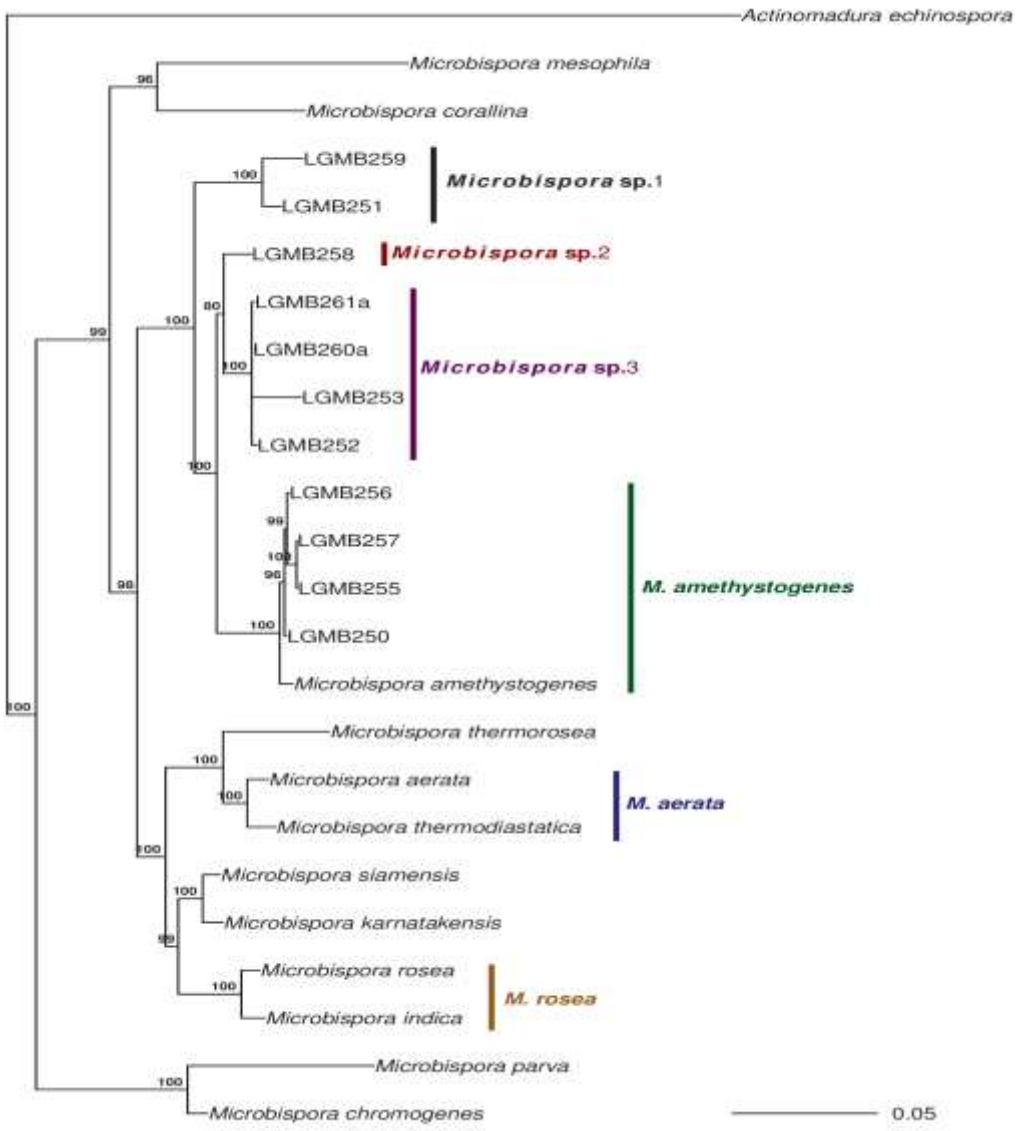

Figure 3. Bayesian phylogeny tree based on *gyr*B and *rpo*B gene concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.05 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

DISCUSSION

The genus *Microbispora* is an important producer of secondary metabolites; species within this genus have similar phenotypes (spore morphology) and analyzes based on 16S rRNA have no phylogenetic support. So, a reliable and powerful discriminating system is

required to classify relationship in this genus, to improve the characterization of species, leading to new species descriptions and increasing the probability of new compounds discovery. This study describes the first Multilocus phylogeny of *Microbispora* species and compare phylogenic trees topology based on four, three, two and single genes with different evolutionary rates.

The four genes we selected are commonly used in the identification of bacteria, and have been used for phylogenetic analyses in actinomycetes (Kim *et al.*, 2004, Dalmasso *et al.*, 2011, Curtis & Meyers, 2012; Busarakam *et al.*, 2014). Two protein-coding genes (the *gyr*B gene which code for the B subunit of DNA gyrase and *rpo*B gene for the B subunit of RNA polymerase) and 23S rRNA were selected to extend the 16S rRNA phylogenetic analysis of the genus *Microbispora*.

In this study, relationships indicated by 16S rRNA analysis was not confirmed by the analyses of concatenated nucleotides, which might be due to the higher levels of sequence identity and thus lower resolution power of this gene. This high level of sequence identity may frequently cause significant changes in the tree topology by the addition or removal of a few sequences. So we can assume that 16S rRNA gene is more appropriate for discrimination at genus level. Limited resolution of 16S rRNA gene sequences was proved before with species when comparing its results with those of genome sequencing (Tamura *et al.*, 2012). Previous studies have been confirmed that sequences of concatenated genes accurately predict genome relatedness and can be used for species-level identification (Tamura *et al.*, 2012; Tambong *et al.*, 2014; Chen *et al.*, 2015). Therefore, we used multiple genes and, as expected, the effect of these problems were reduced considerably (Figure 2).

The results of MLSA did not correlate well with the classification realized by Miyadoh *et al.* (1990); the authors proposed combining the species *M. rosea, M. amethystogenes, M. chromogenes, M. diastatica, M. indica, M. karnatakensis, M. parva,*

M. aerata, *M. thermodiastatica* and *M. thermorosea* as a single taxa *M. rosea*, based on values of DDH range from 37 to 94%, mean 56,46%. The value proposed by Bergeys Manual to delineate species is 70% of DDH, even been correlated in literature it can be higher for some groups (e.g. cryptic species) (Janda and Abbott, 2007). On concatenated sequence *M. thermorosea*, *M. aerata* and *M. thermodiastatica* had 93,53-93,9% nucleotide similarity with *M. rosea* (Supplementary Information, Table S1) and 45-52% of DDH (Miyadoh *et al.*, 1990), these values are low to consider both the same species, as proposed by Miyadoh *et al.* (1990). Other example of inconsistence on the classification realized by Miyadoh *et al.* (1990) is that *M. chromogenes* and *M. parva* had low values of similarity, 87 and 94,87%, with *M. rosea* and 87,26% between themselves on 16S-23S-*gyr*B-*rpo*B concatenated analysis (Supplementary Information, Table S1). Similar incongruence was also observed by Boondaeng *et al.* (2009), the authors proposed that strain *M. amethystogenes* was a separate genomic species from *M. rosea*, supported by genotypic and phenotypic data.

DNA/DNA reassociation is currently recognized as the gold standard in the identification of bacterial species. However, it is difficult to reuse this data for other experiments because experimental errors are commonly found in DDH values and expensive work is necessary. MLSA analyze shown to be a robust and reproducible alternative for DDH (Carro *et al.*, 2012; Curtis & Meyers, 2012).

In our analyses, the phylogenetic tree based on MLSA, most nodes of species branches were well-supported by high probability values, indicate the reliability of nodes and great similarity and evolutionary distance between taxa to validate branch lengths. In the MLSA the species *M. mesophilla*, *M. chromogenes*, *M. parva* and *M. corallina* have the longest branches and were well supported by probability value. A short branch was observed between the species *M rosea* and *M. indica* with 100% probability support, these strains had some minors differences in morphology on ISP4 media (*M. rosea* had a moderated growing

and dark yellow mycelium, and *M. indica* have a good growth and Brown mycelium, Table S13), however showed 97,97% nucleotide similarity, which evidentiate their high correlation. In the group *M. thermodiastatica, M. aerata* and *M. thermorosea,* strain *M. thermorosea* had a longer branch and the species *M. thermodiastatica* and *M. aerata* are more related, sharing 98,75% of similarity. The node of *M. karnatakensis* and *M. siamensis* was supported by 100% probability and have 0,12 of evolutionary distance, with 98,73% of nucleotide similarity. Despite of high similarity on concatenated analysis, these species have morphologic evidences for separation, *M. siamensis* produce a green pigment on ISP3 media, what is not observer for *M. karnatakensis* (Table S13); *M. siamensis* also grow better at 55°C and have DNA-DNA hybridization value of 19-46% with *Microbispora* species (Boondaeng *et al.,* 2009). The second cluster was formed by a clade with type strain *M. amethystogenes* and isolates LGMB255, LGMB256, LGMB257, LGMB250 strongly related sharing 98,79 to 99,73% similarity, so the endophytic isolates were classified as *M. amethystogenes*. This is the first report of *M. amethystogenes* isolated as endophytic and also producing secondary metabolites with antibacterial, antitumor and antioxidant activities (Savi *et al.,* 2015). The three other clades are formed by the endophytic isolates and supported by 100% probability value. LGMB251 and LGMB259 was characterized as *Microbispora* sp. 1, with 97,63% similarity and 0,015 of evolutionary distance. Strain LGMB259 was correlated with the production of secondary metabolites that showed biological activities (Savi *et al.,* 2015), as the compound 1-Vinyl-*β*-carboline-3-carboxylic acid that displayed antimicrobial and cytotoxicity activities (Savi *et al.,* 2014). Strain LGMB258 was characterized as *Microbispora* sp. 2, this strain is correlated with strains LGMB251 and LGMB259, but is well separated of *Microbispora* sp. 1 cluster, with 0,022 evolutionary distance and 94,02-92,54% of similarity values. The last clade was formed by LGMB252, LGMB253, LGMB260a and LGMB261a supported by 100% probability support, with 99,19 to 99,95%

similarity and 0,000 to 0,008 of divergence, and was classified as *Microbispora* sp. 3. The MLSA showed evidence that the endophytic isolates belong to clusters *Microbispora* sp. 1, *Microbispora* sp. 2 and *Microbispora* sp. 3 are new species, and DNA-DNA hybridization and morphologic tests are necessary to describe these species.

Single gene phylogenies showed various degrees of resolution, *gyr*B yield the best separation of the reference strains, but with lower probability value and shorter branches than MLSA. The protein coding genes exhibited higher levels of variations than rRNA genes, and notably the 23S rRNA gene was more variable than 16S rRNA gene. This study also showed that the concatenate genes *gyr*B-*rpo*B gave highly congruent representation for the genus *Microbispora*, yielded the best resolution topology, probability values were greatly improved than MLSA, proving to be a robust method for the differentiation of most *Microbispora* species and appear to offer indicative method for the placement of individual strains.

Based on careful analysis of phylogenetic trees, morphologic characteristics, evolutionary distance and nucleotide similarity of concatenated genes *gyr*B-*rpo*B and MLSA, we suggested that strains *M. amethystogenes, M. chromogenes, M. karnatakensis, M. parva, M. aerata, M. thermodiastatica* and *M. thermorosea* merits species status distinct from *M. rosea*, rather than being a member of the latter species as proposed by Miyadoh *et al.* (1990). However, strain *M. indica* showed high similarity with *M. rosea* (Figure 2, Supp. Information, Table S1) and are the same species as proposed by Miyadoh *et al.* (1990). *M. aerata* and M. *thermodiastatica* also showed high values of similarity and short branches in MLSA analyzes and probably are the same species (Figure 2, Supp. Information, Table S1). We also propose the value of 98,0% nucleotide similarity on *gyr*B-*rpo*B loci for a cut-off value for species delineation in the genus *Microbispora*.

In conclusion, the 16S rRNA gene sequence is extremely limiting in the discrimination of species in the *Microbispora* genus; by analyses of multilocus sequence we

suggest that species *M. amethystogenes*, *M. chromogenes*, *M. karnatakensis*, *M. parva*, *M. aerata*, *M. thermodiastatica* and *M. thermorosea* are distinct from *M. rosea*; however *M. aerata* and M. *thermodiastatica* probably are the same species, as well *M. indica* and *M. rosea*. Endophytic isolates from a Brazilian Medicinal plant *V. divergens* belongs the clusters *Microbispora* sp. 1, *Microbispora* sp. 2 and *Microbispora* sp. 3 are different from the *Microbispora* species previous described and futures studies are required to describe these species. It is also proposed the concatenated analyses of *gyr*B-*rpo*B genes as a useful alternative to DNA–DNA hybridization for the identification and phylogenetic analysis in the *Microbispora* genus, and values less than 98% to characterization and determines relationship at species level.

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Supplementary Information

Multilocus Sequence Analysis of the Genus *Microbispora*

SAVI, D.C. [1]; ALUIZIO, R.. [2]; GLIENKE, C. [2]

Affiliations: [1]Department of Pathology, Universidade Federal do Paraná, Av. Coronel Francisco Heráclito dos Santos, 210. CEP: 81531-970, Curitiba, PR, Brazil. [2]Department of Genetics, Universidade Federal do Parana, PO.BOX 19071. CEP: 81531-970, Curitiba, PR, Brazil.

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nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

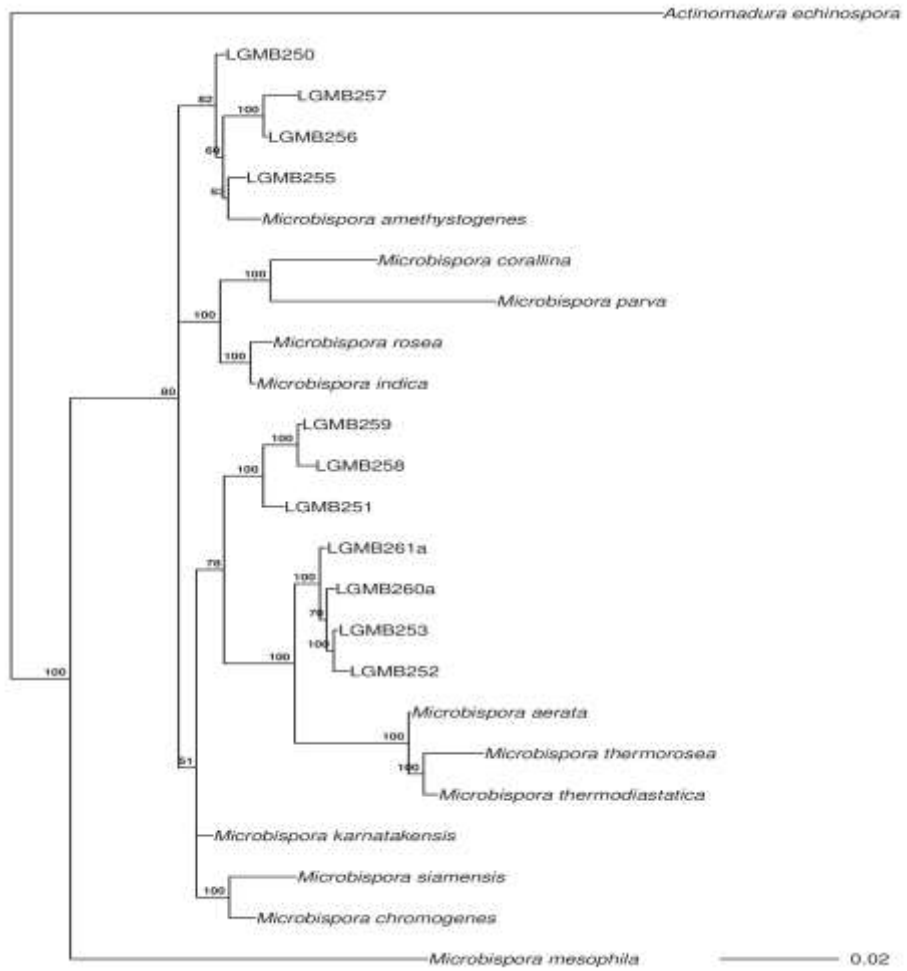

Figure S1. Bayesian phylogeny tree based on 16S rRNA and 23S rRNA genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.02 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

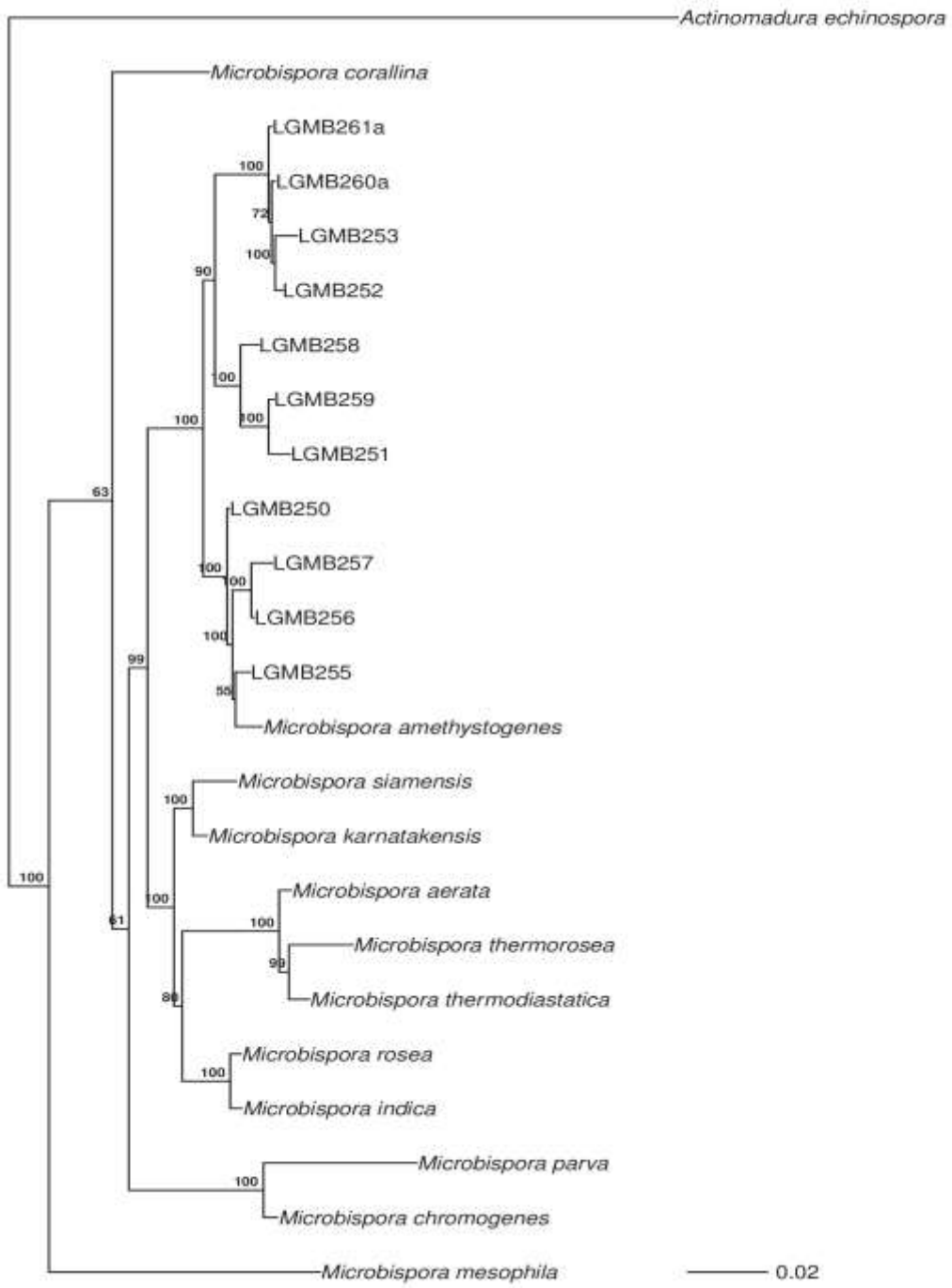

Figure S2. Bayesian phylogeny tree based on 16S rRNA, 23S rRNA and *gyr*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.02 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

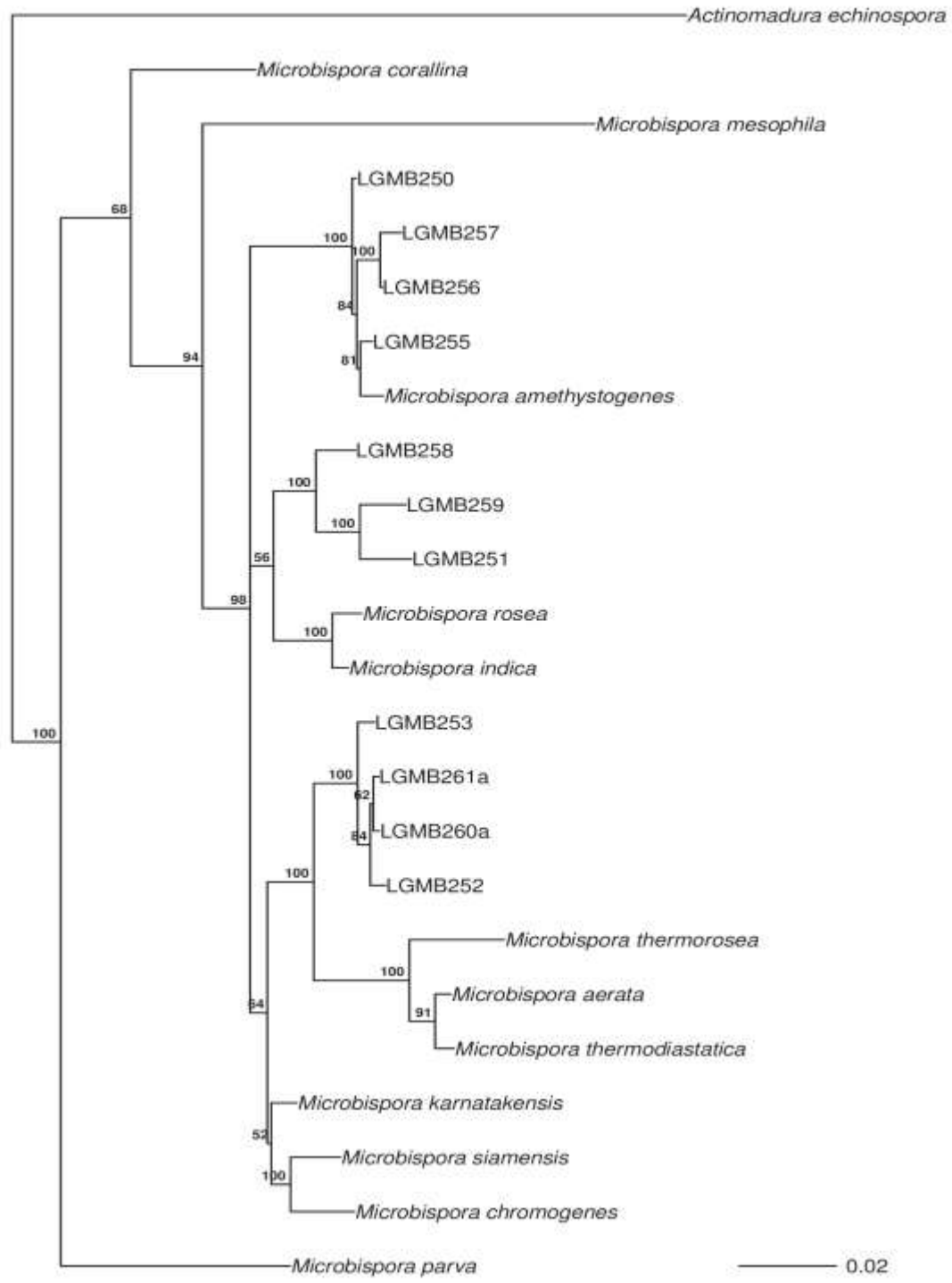

Figure S3. Bayesian phylogeny tree based on 16S rRNA, 23S rRNA and *rpo*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.02 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

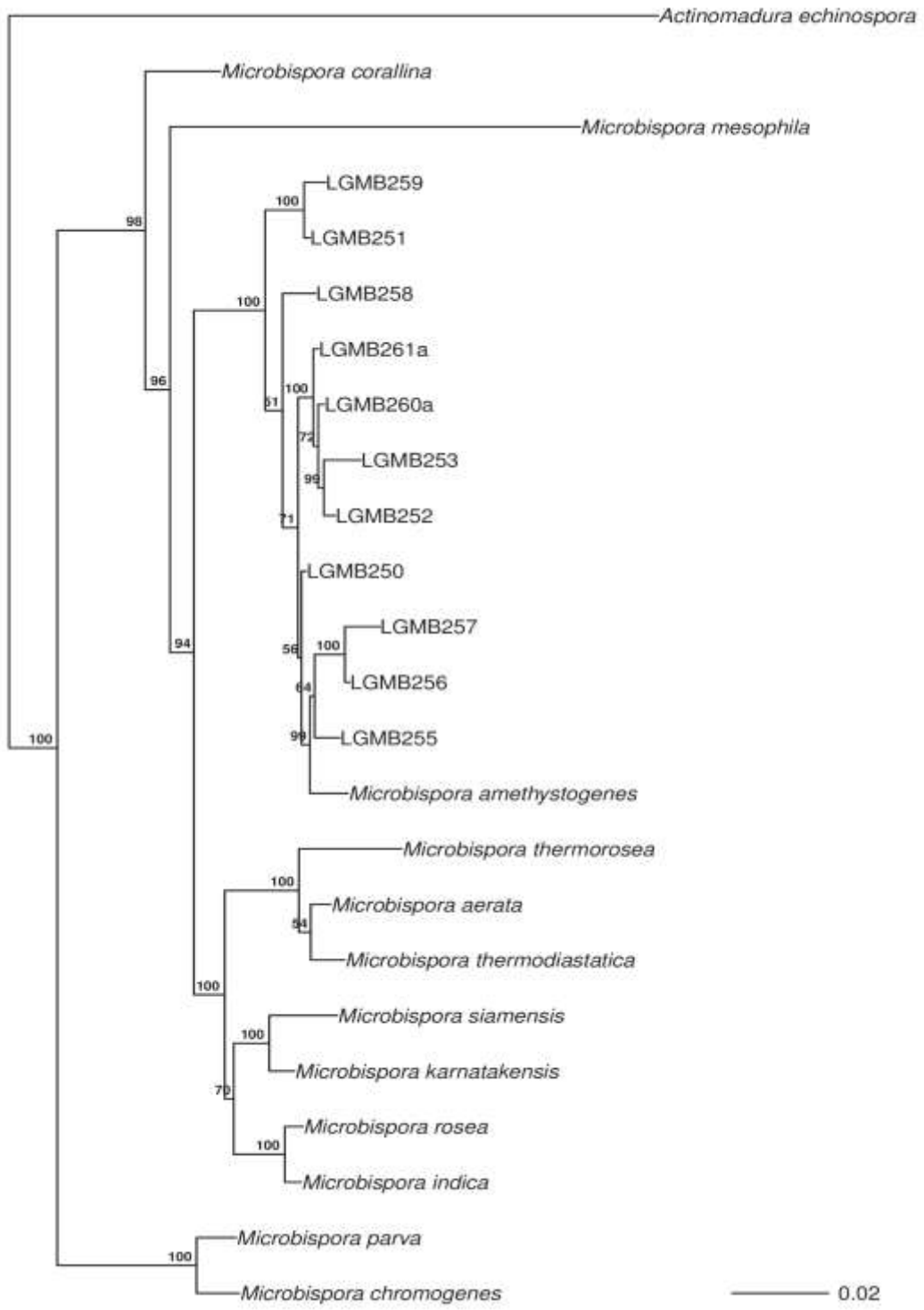

Figure S4. Bayesian phylogeny tree based on 16S rRNA and *gyr*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.02 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

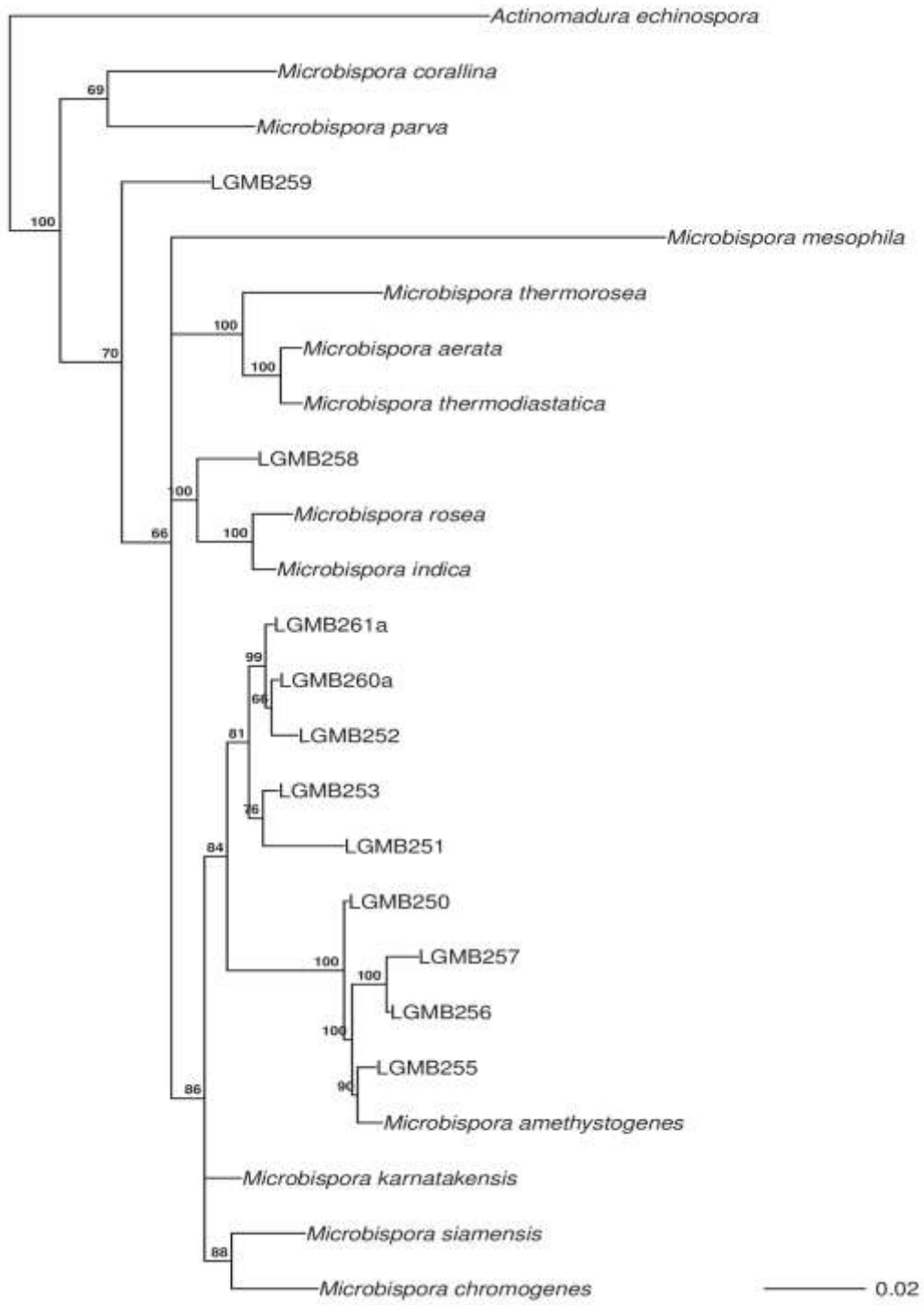

Figure S5. Bayesian phylogeny tree based on 16S rRNA and *rpo*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.02 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

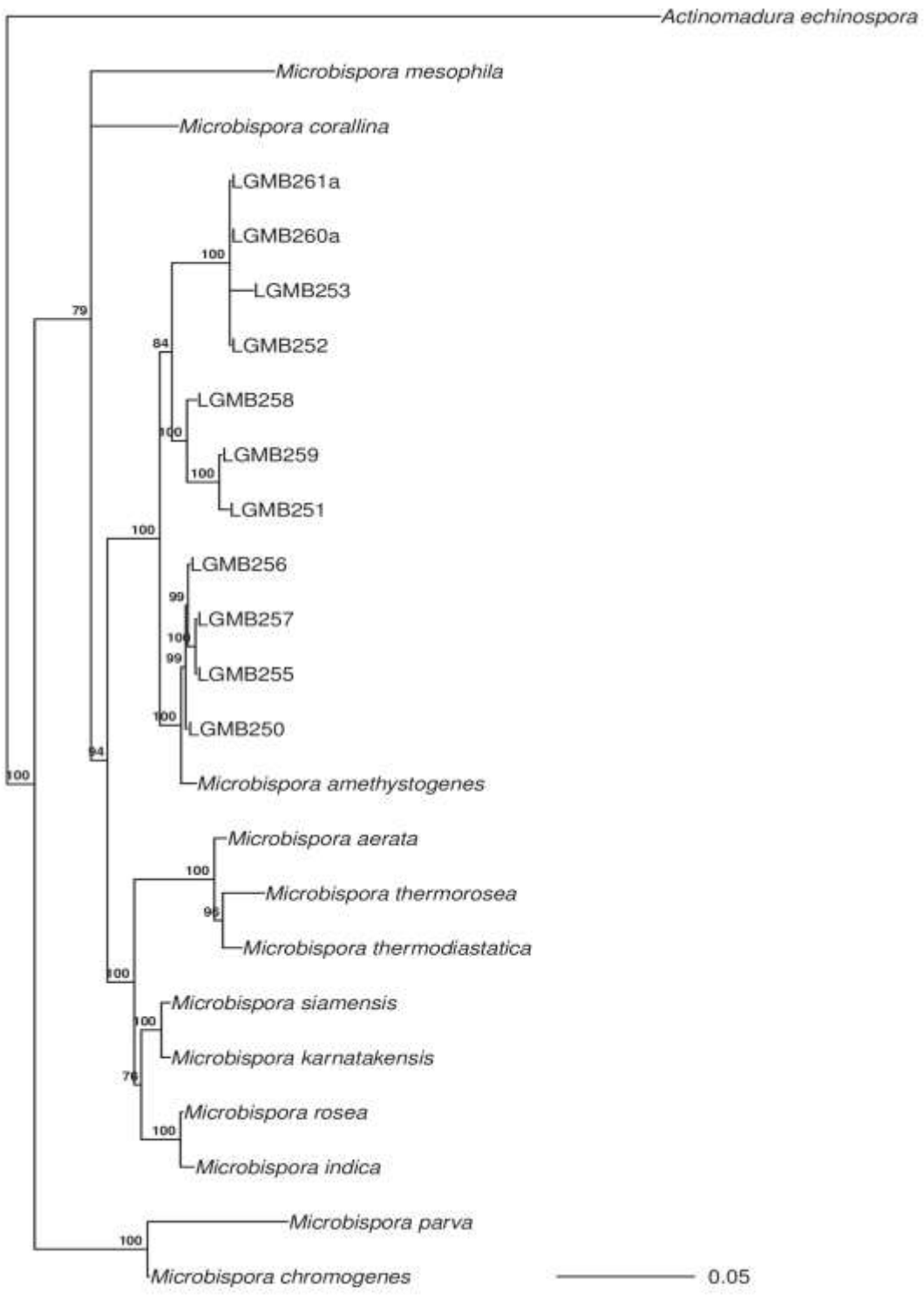

Figure S6. Bayesian phylogeny tree based on 23S rRNA and *gyr*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.04 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

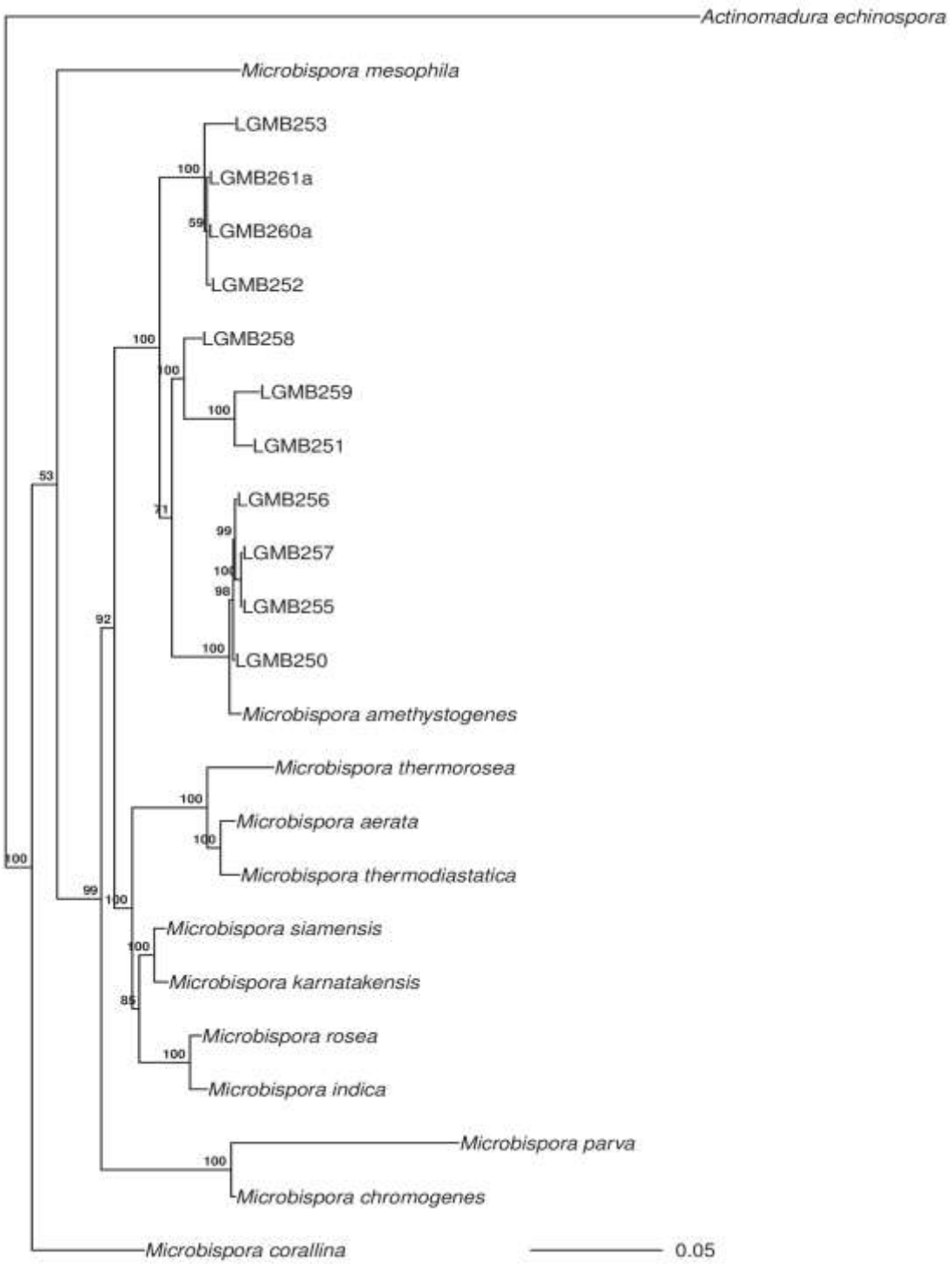

Figure S7. Bayesian phylogeny tree based on 23S rRNA, *gyr*B and *rpo*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.05 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

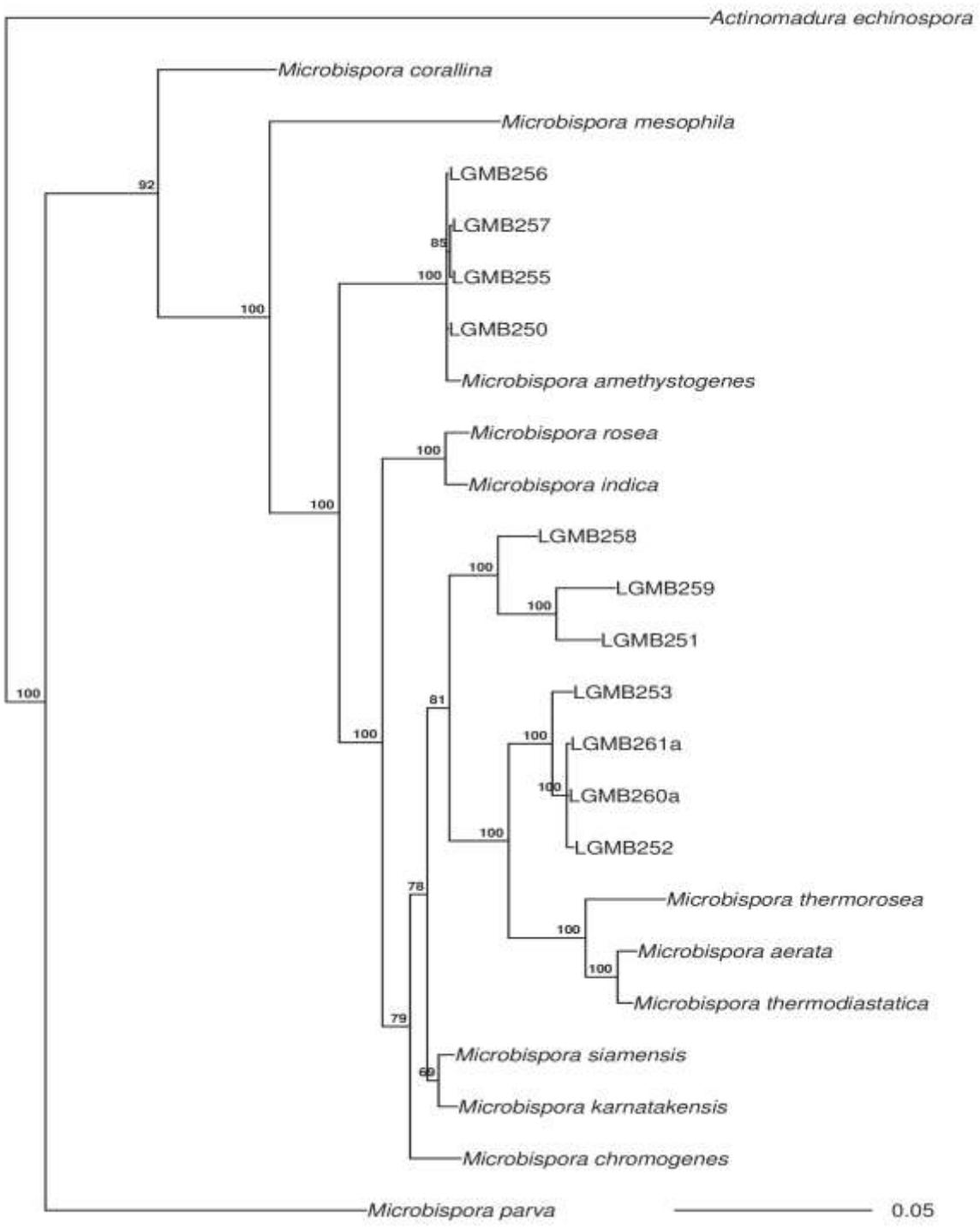

Figure S8. Bayesian phylogeny tree based on 23S rRNA and *rpo*B genes concatenated sequences, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.03 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

Figure S9. Bayesian phylogeny tree based on 23S rRNA gene sequence, showing the relationship between 11 Microbispora isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.05 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

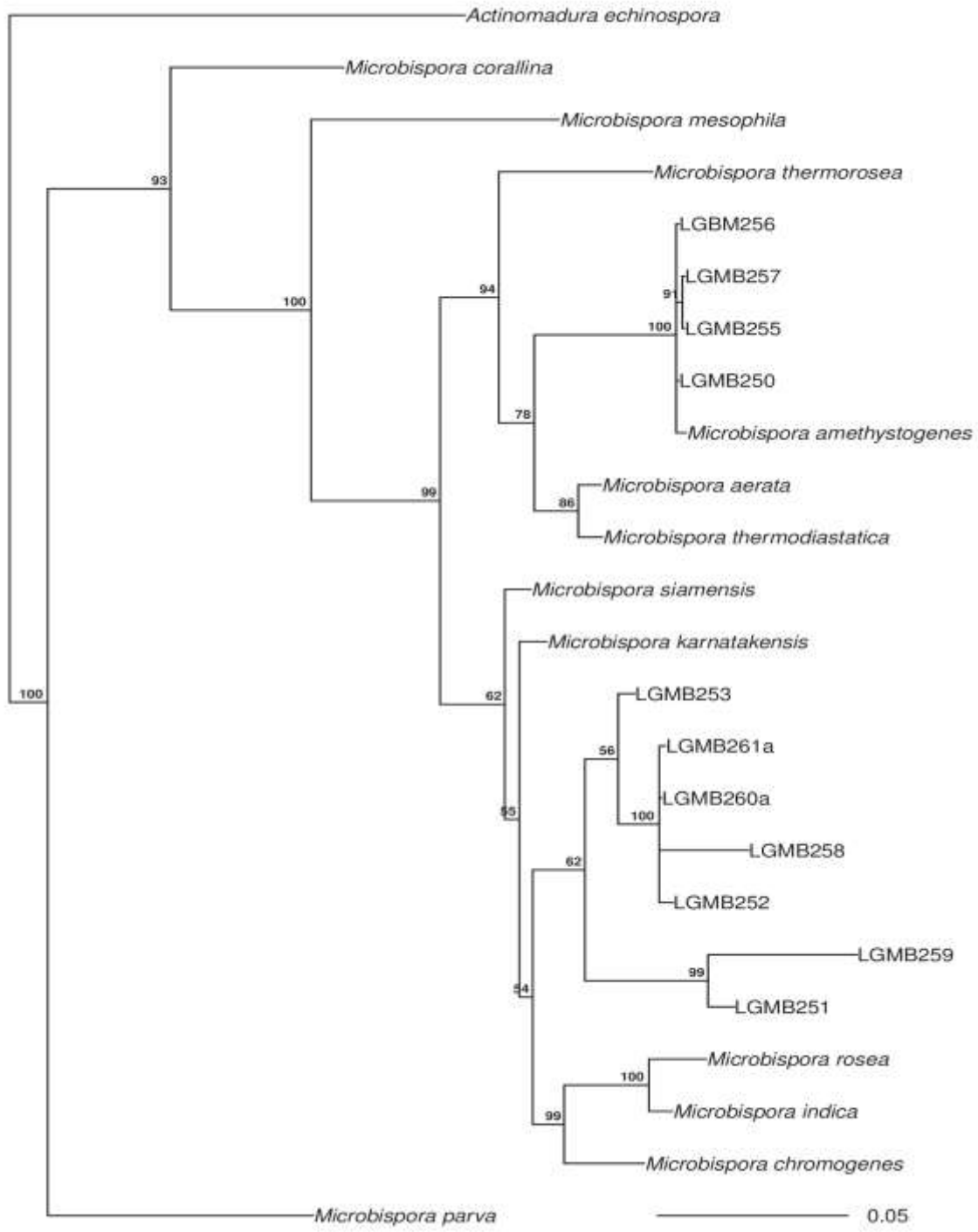

Figure S10. Bayesian phylogeny tree based on *rpo*B gene sequence, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.05 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees

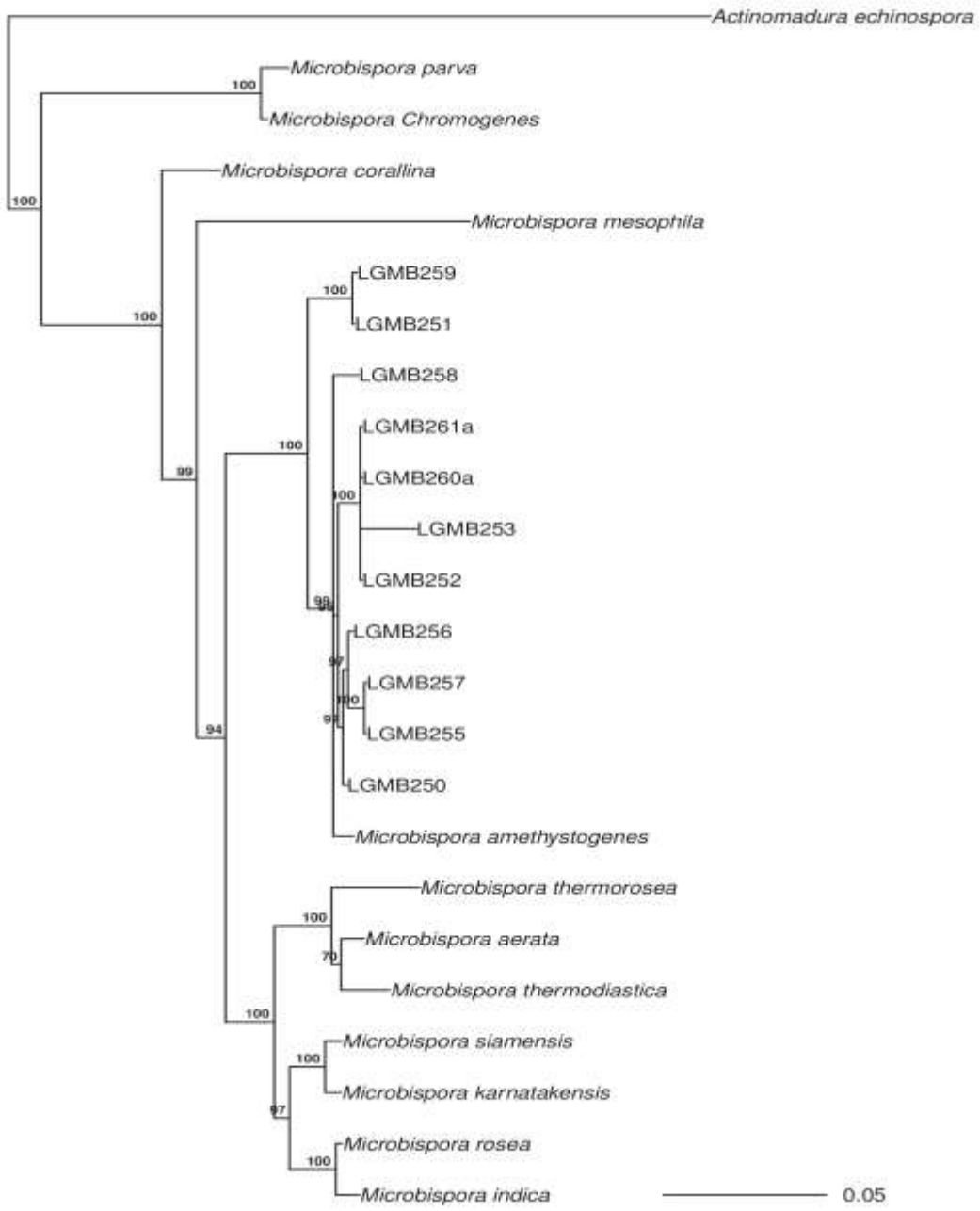

Figure S11. Bayesian phylogeny tree based on *gyr*B gene sequence, showing the relationship between 11 *Microbispora* isolates and the 12 recognized *Microbispora* species. *Actinomadura echinospora* was used as outgroup. Bar, 0.02 substitutions per nucleotide position. Values on the branch indicate Bayesian posterior probabilities expressed as a percentage of the trees.

Table S1. Similarity percentage matrix for the genes 16S rRNA, 23S rRNA, *gyr*B and *rpo*B

	Microbispora chromogenes	***Microbispora indica***	***Microbispora karnatakensis***	***Microbispora parva***	***Microbispora thermodiastatica***	***Microbispora thermorosea***	***Microbispora amethystogenes***	***Microbispora corallina***	***Microbispora mesophila***	***Microbispora aerata***	***Microbispora rosea***	***Microbispora siamensis***
Microbispora chromogenes		95,59	96,31	87,26	93,66	92,79	92,42	89,11	89,89	93,16	94,87	96,07
Microbispora indica	95,59		96,07	86,73	93,53	92,17	92,42	89,1	89,63	93,28	97,94	95,83
Microbispora karnatakensis	96,31	96,07		88,19	95,59	93,41	92,67	89,63	90,27	95,35	95,35	98,41
Microbispora parva	87,26	86,73	88,19		88,58	87,38	86,31	89,5	86,86	88,58	87	87,93
Microbispora thermodiastatica	93,66	93,53	95,59	88,58		94,26	94,74	89,11	90,91	98,75	93,9	95,59
Microbispora thermorosea	92,79	92,17	93,41	87,38	94,26		94,02	86,72	89,89	94,14	93,53	93,41
Microbispora amethystogenes	92,42	92,42	92,67	86,31	94,74	94,02		87,92	89,5	95,1	93,41	93,41
Microbispora corallina	89,11	89,1	89,63	89,5	89,11	86,72	87,92		89,76	88,85	89,37	89,62
Microbispora mesophila	89,89	89,63	90,27	86,86	90,91	89,89	89,5	89,76		90,27	90,02	90,53
Microbispora aerata	93,16	93,28	95,35	88,58	98,75	94,14	95,1	88,85	90,27		93,65	95,59
Microbispora rosea	94,87	97,94	95,35	87	93,9	93,53	93,41	89,37	90,02	93,65		95,59
Microbispora siamensis	96,07	95,83	98,41	87,93	95,59	93,41	93,41	89,62	90,53	95,59	95,59	
LGMB250	92,67	92,42	92,92	86,58	94,98	94,26	99,77	88,19	89,76	95,34	93,41	93,41
LGMB251	94,02	92,55	94,51	88,59	93,54	91,16	91,29	90,27	89,76	92,92	92,05	94,02
LGMB252	95,47	94,75	96,19	87,4	94,63	93,04	93,54	88,45	89,37	94,63	94,75	97,36
LGMB253	95,83	94,99	97,13	87,27	94,27	92,92	92,67	88,58	89,24	94,02	94,63	96,78
LGMB255	92,67	92,42	92,79	86,58	94,86	94,14	99,66	88,19	89,76	95,22	93,41	93,29
LGMB256	92,67	92,42	92,92	86,58	94,98	94,26	99,77	88,19	89,76	95,34	93,41	93,41
LGMB257	92,67	92,42	92,79	86,58	94,86	94,14	99,66	88,19	89,76	95,22	93,41	93,29
LGMB258	95,47	95,47	95,59	87,4	94,26	93,41	94,27	88,45	89,76	94,51	95,47	96,31
LGMB259	92,92	92,17	93,29	89,11	93,04	89,63	90,53	89,49	89,63	92,3	91,79	93,54
LGMB260a	95,59	94,87	96,54	87,26	94,75	93,41	93,9	88,32	89,5	94,75	95,11	97,48
LGMB261a	95,71	94,75	96,42	87,13	94,63	93,29	93,78	88,19	89,37	94,63	94,99	97,36
Actinomadura echinospora	86,04	86,03	86,18	87,1	86,84	87,1	86,16	86,99	84,94	86,71	86,57	87,39

Table S1. Similarity percentage matrix for the genes 16S rRNA, 23S rRNA, *gyr*B and *rpo*B (continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGBM256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora chromogenes	92,67	94,02	95,47	95,83	92,67	92,67	92,67	95,47	92,92	95,59	95,71	86,04
Microbispora indica	92,42	92,55	94,75	94,99	92,42	92,42	92,42	95,47	92,17	94,87	94,75	86,03
Microbispora karnatakensis	92,92	94,51	96,19	97,13	92,79	92,92	92,79	95,59	93,29	96,54	96,42	86,18
Microbispora parva	86,58	88,59	87,4	87,27	86,58	86,58	86,58	87,4	89,11	87,26	87,13	87,1
Microbispora thermodiastatica	94,98	93,54	94,63	94,27	94,86	94,98	94,86	94,26	93,04	94,75	94,63	86,84
Microbispora thermorosea	94,26	91,16	93,04	92,92	94,14	94,26	94,14	93,41	89,63	93,41	93,29	87,1
Microbispora amethystogenes	99,77	91,29	93,54	92,67	99,66	99,77	99,66	94,27	90,53	93,9	93,78	86,16
Microbispora corallina	88,19	90,27	88,45	88,58	88,19	88,19	88,19	88,45	89,49	88,32	88,19	86,99
Microbispora mesophila	89,76	89,76	89,37	89,24	89,76	89,76	89,76	89,76	89,63	89,5	89,37	84,94
Microbispora aerata	95,34	92,92	94,63	94,02	95,22	95,34	95,22	94,51	92,3	94,75	94,63	86,71
Microbispora rosea	93,41	92,05	94,75	94,63	93,41	93,41	93,41	95,47	91,79	95,11	94,99	86,57
Microbispora siamensis	93,41	94,02	97,36	96,78	93,29	93,41	93,29	96,31	93,54	97,48	97,36	87,39
LGMB250		91,55	93,54	92,92	99,89	100	99,89	94,27	90,53	93,9	93,78	86,16
LGMB251	91,55		94,63	95,71	91,42	91,55	91,42	94,02	95,83	94,75	94,63	85,09
LGMB252	93,54	94,63		98,06	93,41	93,54	93,41	97,36	92,79	99,66	99,55	85,91
LGMB253	92,92	95,71	98,06		92,8	92,92	92,8	96,07	92,67	98,41	98,29	85,36
LGMB255	99,89	91,42	93,41	92,8		99,89	100	94,15	90,4	93,78	93,66	86,03
LGMB256	100	91,55	93,54	92,92	99,89		99,89	94,27	90,53	93,9	93,78	86,16
LGMB257	99,89	91,42	93,41	92,8	100	99,89		94,15	90,4	93,78	93,66	86,03
LGMB258	94,27	94,02	97,36	96,07	94,15	94,27	94,15		92,54	97,71	97,6	85,76
LGMB259	90,53	95,83	92,79	92,67	90,4	90,53	90,4	92,54		92,67	92,79	86,45
LGMB260a	93,9	94,75	99,66	98,41	93,78	93,9	93,78	97,71	92,67		99,89	85,63
LGMB261a	93,78	94,63	99,55	98,29	93,66	93,78	93,66	97,6	92,79	99,89		85,49
Actinomadura echinospora	86,16	85,09	85,91	85,36	86,03	86,16	86,03	85,76	86,45	85,63	85,49	

Table S2. Similarity percentage matrix for the genes *gyr*B and *rpo*B

	Microbispora chromogenes	***Microbispora indica***	***Microbispora karnatakensis***	***Microbispora parva***	***Microbispora thermodiastatica***	***Microbispora thermorosea***	***Microbispora amethystogenes***	***Microbispora corallina***	***Microbispora mesophila***	***Microbispora aerata***	***Microbispora rosea***	***Microbispora siamensis***
Microbispora chromogenes		92,24	92,72	92,73	91,11	90,54	90,76	89,25	88,37	91,39	92,1	92,52
Microbispora indica	92,24		96,05	87,11	94,1	93,08	93,14	91,25	90,33	94,51	98,41	95,86
Microbispora karnatakensis	92,72	96,05		88	95,66	94,11	93,35	91,68	90,83	95,86	96,06	98,54
Microbispora parva	92,73	87,11	88		88,14	87,47	87,4	89,47	86,36	88,73	87,56	87,78
Microbispora thermodiastatica	91,11	94,1	95,66	88,14		95,12	94,17	91,18	90,76	98,34	94,71	95,59
Microbispora thermorosea	90,54	93,08	94,11	87,47	95,12		93,83	89,9	90,19	95,19	94,17	94,04
Microbispora amethystogenes	90,76	93,14	93,35	87,4	94,17	93,83		90,76	90,69	94,84	94,03	93,63
Microbispora corallina	89,25	91,25	91,68	89,47	91,18	89,9	90,76		90,26	91,68	91,75	91,89
Microbispora mesophila	88,37	90,33	90,83	86,36	90,76	90,19	90,69	90,26		90,76	90,76	91,05
Microbispora aerata	91,39	94,51	95,86	88,73	98,34	95,19	94,84	91,68	90,76		95,05	95,92
Microbispora rosea	92,1	98,41	96,06	87,56	94,71	94,17	94,03	91,75	90,76	95,05		96,12
Microbispora siamensis	92,52	95,86	98,54	87,78	95,59	94,04	93,63	91,89	91,05	95,92	96,12	
LGMB250	90,9	93,28	93,63	87,55	94,44	93,97	99,49	90,83	90,83	95,11	94,03	93,76
LGMB251	91,33	92,94	94,11	88,3	93,15	92,03	94,65	91,68	90,62	93,28	92,94	93,7
LGMB252	92,52	94,58	95,66	87,93	94,24	93,15	95,86	90,83	90,76	94,72	94,92	96,12
LGMB253	92,52	95,05	96,12	87,63	93,97	93,15	94,99	90,97	90,62	94,31	95,19	95,86
LGMB255	90,76	93,14	93,28	87,4	94,1	93,63	99,18	90,68	90,83	94,78	93,9	93,56
LGMB256	90,76	93,21	93,49	87,4	94,3	93,83	99,43	90,69	90,76	94,98	93,97	93,63
LGMB257	90,76	93,14	93,28	87,4	94,1	93,63	99,18	90,68	90,83	94,78	93,9	93,56
LGMB258	92,66	95,05	95,32	88,08	94,51	93,7	96,26	90,9	90,76	94,98	95,39	95,59
LGMB259	90,62	92,66	93,35	88,52	92,8	91,12	94,17	91,18	90,48	92,87	92,73	93,35
LGMB260a	92,59	94,65	95,86	87,85	94,31	93,35	96,06	90,76	90,83	94,78	95,12	96,19
LGMB261a	92,66	94,58	95,79	87,78	94,24	93,28	95,99	90,68	90,76	94,72	95,05	96,12
Actinomadura echinospora	83,34	83,01	82,85	83,79	83,32	83,23	82,83	83,64	82,54	83,72	83,64	83,33

Table S2. Similarity percentage matrix for the genes *gyr*B and *rpo*B (continued)

	LGMB250	LGMB251	LGMB252	LGMB253	LGMB255	LGMB256	LGMB257	LGMB258	LGMB259	LGMB260a	LGMB261a	*Actinomadura echinospora*
Microbispora chromogenes	90,9	91,33	92,52	92,52	90,76	90,76	90,76	92,66	90,62	92,59	92,66	83,34
Microbispora indica	93,28	92,94	94,58	95,05	93,14	93,21	93,14	95,05	92,66	94,65	94,58	83,01
Microbispora karnatakensis	93,63	94,11	95,66	96,12	93,28	93,49	93,28	95,32	93,35	95,86	95,79	82,85
Microbispora parva	87,55	88,3	87,93	87,63	87,4	87,4	87,4	88,08	88,52	87,85	87,78	83,79
Microbispora thermodiastatica	94,44	93,15	94,24	93,97	94,1	94,3	94,1	94,51	92,8	94,31	94,24	83,32
Microbispora thermorosea	93,97	92,03	93,15	93,15	93,63	93,83	93,63	93,7	91,12	93,35	93,28	83,23
Microbispora amethystogenes	99,49	94,65	95,86	94,99	99,18	99,43	99,18	96,26	94,17	96,06	95,99	82,83
Microbispora corallina	90,83	91,68	90,83	90,97	90,68	90,69	90,68	90,9	91,18	90,76	90,68	83,64
Microbispora mesophila	90,83	90,62	90,76	90,62	90,83	90,76	90,83	90,76	90,48	90,83	90,76	82,54
Microbispora aerata	95,11	93,28	94,72	94,31	94,78	94,98	94,78	94,98	92,87	94,78	94,72	83,72
Microbispora rosea	94,03	92,94	94,92	95,19	93,9	93,97	93,9	95,39	92,73	95,12	95,05	83,64
Microbispora siamensis	93,76	93,7	96,12	95,86	93,56	93,63	93,56	95,59	93,35	96,19	96,12	83,33
LGMB250		94,65	95,99	95,26	99,56	99,87	99,56	96,39	94,04	96,19	96,12	82,59
LGMB251	94,65		96,12	96,32	94,31	94,58	94,31	95,79	97,63	96,19	96,12	81,89
LGMB252	95,99	96,12		98,47	95,79	95,92	95,79	97,76	95,05	99,81	99,75	82,61
LGMB253	95,26	96,32	98,47		95,05	95,19	95,05	96,65	94,58	98,67	98,6	82,53
LGMB255	99,56	94,31	95,79	95,05		99,62	100	95,92	93,7	95,99	95,92	82,36
LGMB256	99,87	94,58	95,92	95,19	99,62		99,62	96,26	93,97	96,12	96,06	82,51
LGMB257	99,56	94,31	95,79	95,05	100	99,62		95,92	93,7	95,99	95,92	82,36
LGMB258	96,39	95,79	97,76	96,65	95,92	96,26	95,92		94,92	97,96	97,89	82,6
LGMB259	94,04	97,63	95,05	94,58	93,7	93,97	93,7	94,92		94,99	95,05	82,61
LGMB260a	96,19	96,19	99,81	98,67	95,99	96,12	95,99	97,96	94,99		99,94	82,45
LGMB261a	96,12	96,12	99,75	98,6	95,92	96,06	95,92	97,89	95,05	99,94		82,37
Actinomadura echinospora	82,59	81,89	82,61	82,53	82,36	82,51	82,36	82,6	82,61	82,45	82,37	

Table S3. Similarity percentage matrix for the gene *gyr*B

	Microbispora chromogenes	*Microbispora indica*	*Microbispora karnatakensis*	*Microbispora parva*	*Microbispora thermodiastatica*	*Microbispora thermorosea*	*Microbispora amethystogenes*	*Microbispora corallina*	*Microbispora mesophila*	*Microbispora aerata*	*Microbispora rosea*	*Microbispora siamensis*
Microbispora chromogenes		87,74	87,91	99,14	87,75	87,58	88,6	89,42	86,41	89,08	88,42	87,76
Microbispora indica	87,74		96,04	87,57	94,82	94,21	94,05	93,9	91,22	96,04	98,99	95,89
Microbispora karnatakensis	87,91	96,04		87,74	95,74	94,98	94,2	94,2	91,54	96,49	96,94	98,7
Microbispora parva	99,14	87,57	87,74		87,58	87,58	88,77	89,42	85,72	88,91	88,25	87,59
Microbispora thermodiastatica	87,75	94,82	95,74	87,58		96,19	93,43	93,74	90,57	97,83	95,74	95,59
Microbispora thermorosea	87,58	94,21	94,98	87,58	96,19		93,59	93,74	90,57	96,49	94,98	94,82
Microbispora amethystogenes	88,6	94,05	94,2	88,77	93,43	93,59		94,2	92,18	94,52	94,82	93,9
Microbispora corallina	89,42	93,9	94,2	89,42	93,74	93,74	94,2		90,89	95,13	94,67	94,67
Microbispora mesophila	86,41	91,22	91,54	85,72	90,57	90,57	92,18	90,89		91,38	91,7	91,7
Microbispora aerata	89,08	96,04	96,49	88,91	97,83	96,49	94,52	95,13	91,38		96,79	96,34
Microbispora rosea	88,42	98,99	96,94	88,25	95,74	94,98	94,82	94,67	91,7	96,79		96,79
Microbispora siamensis	87,76	95,89	98,7	87,59	95,59	94,82	93,9	94,67	91,7	96,34	96,79	
LGMB250	88,6	94,36	94,51	88,76	93,74	93,59	99,14	94,05	92,18	94,82	94,82	94,21
LGMB251	87,77	93,43	93,59	87,93	92,65	93,12	98,7	93,43	91,7	93,74	94,05	93,28
LGMB252	88,6	94,36	94,98	88,59	93,74	93,28	98,7	93,74	92,49	94,82	95,12	94,52
LGMB253	88,09	95,13	94,82	88,09	93,59	93,43	97,83	93,89	92,33	94,67	95,89	94,67
LGMB255	88,27	94,05	93,9	88,43	93,12	92,97	98,56	93,74	92,18	94,21	94,51	93,9
LGMB256	88,26	94,21	94,2	88,43	93,43	93,28	98,99	93,74	92,02	94,52	94,67	93,9
LGMB257	88,27	94,05	93,9	88,43	93,12	92,97	98,56	93,74	92,18	94,21	94,51	93,9
LGMB258	88,92	94,51	94,97	88,93	94,82	94,06	98,7	93,89	92,02	95,58	95,28	94,67
LGMB259	87,6	93,28	93,43	87,77	92,49	92,96	98,56	93,27	91,54	93,59	93,9	93,12
LGMB260a	88,6	94,36	94,98	88,59	93,74	93,28	98,7	93,74	92,49	94,82	95,12	94,52
LGMB261a	88,6	94,36	94,98	88,59	93,74	93,28	98,7	93,74	92,49	94,82	95,12	94,52
Actinomadura echinospora	79,75	78,97	78,37	79,38	78,6	78,01	78,37	79,15	79,36	79,72	79,74	77,83

Table S3. Similarity percentage matrix for the gene *gyr*B (continued)

	LGMB250	LGMB251	LGMB252	LGMB253	LGMB255	LGMB256	LGMB257	LGMB258	LGMB259	LGMB260a	LGMB261a	*Actinomadura echinospora*
Microbispora chromogenes	88,6	87,77	88,6	88,09	88,27	88,26	88,27	88,92	87,6	88,6	88,6	79,75
Microbispora indica	94,36	93,43	94,36	95,13	94,05	94,21	94,05	94,51	93,28	94,36	94,36	78,97
Microbispora karnatakensis	94,51	93,59	94,98	94,82	93,9	94,2	93,9	94,97	93,43	94,98	94,98	78,37
Microbispora parva	88,76	87,93	88,59	88,09	88,43	88,43	88,43	88,93	87,77	88,59	88,59	79,38
Microbispora thermodiastatica	93,74	92,65	93,74	93,59	93,12	93,43	93,12	94,82	92,49	93,74	93,74	78,6
Microbispora thermorosea	93,59	93,12	93,28	93,43	92,97	93,28	92,97	94,06	92,96	93,28	93,28	78,01
Microbispora amethystogenes	99,14	98,7	98,7	97,83	98,56	98,99	98,56	98,7	98,56	98,7	98,7	78,37
Microbispora corallina	94,05	93,43	93,74	93,89	93,74	93,74	93,74	93,89	93,27	93,74	93,74	79,15
Microbispora mesophila	92,18	91,7	92,49	92,33	92,18	92,02	92,18	92,02	91,54	92,49	92,49	79,36
Microbispora aerata	94,82	93,74	94,82	94,67	94,21	94,52	94,21	95,58	93,59	94,82	94,82	79,72
Microbispora rosea	94,82	94,05	95,12	95,89	94,51	94,67	94,51	95,28	93,9	95,12	95,12	79,74
Microbispora siamensis	94,21	93,28	94,52	94,67	93,9	93,9	93,9	94,67	93,12	94,52	94,52	77,83
LGMB250		98,41	98,99	98,12	99,14	99,71	99,14	98,99	98,27	98,99	98,99	77,79
LGMB251	98,41		97,97	97,09	97,82	98,26	97,82	97,97	99,86	97,97	97,97	77,61
LGMB252	98,99	97,97		98,99	98,7	98,85	98,7	98,27	97,83	100	100	78,18
LGMB253	98,12	97,09	98,99		97,83	97,97	97,83	97,38	96,94	98,99	98,99	78,76
LGMB255	99,14	97,82	98,7	97,83		99,28	100	98,12	97,68	98,7	98,7	77,42
LGMB256	99,71	98,26	98,85	97,97	99,28		99,28	98,7	98,12	98,85	98,85	77,61
LGMB257	99,14	97,82	98,7	97,83	100	99,28		98,12	97,68	98,7	98,7	77,42
LGMB258	98,99	97,97	98,27	97,38	98,12	98,7	98,12		97,83	98,27	98,27	78,36
LGMB259	98,27	99,86	97,83	96,94	97,68	98,12	97,68	97,83		97,83	97,83	77,42
LGMB260a	98,99	97,97	100	98,99	98,7	98,85	98,7	98,27	97,83		100	78,18
LGMB261a	98,99	97,97	100	98,99	98,7	98,85	98,7	98,27	97,83	100		78,18
Actinomadura echinospora	77,79	77,61	78,18	78,76	77,42	77,61	77,42	78,36	77,42	78,18	78,18	

Table S4. Similarity percentage matrix for the gene *rpo*B

	Microbispora chromogenes	*Microbispora indica*	*Microbispora karnatakensis*	*Microbispora parva*	*Microbispora thermodiastatica*	*Microbispora Thermorosea*	*Microbispora amethystogenes*	*Microbispora corallina*	*Microbispora mesophila*	*Microbispora aerata*	*Microbispora rosea*	*Microbispora siamensis*
Microbispora chromogenes		95,59	96,31	87,26	93,66	92,79	92,42	89,11	89,89	93,16	94,87	96,07
Microbispora indica	95,59		96,07	86,73	93,53	92,17	92,42	89,1	89,63	93,28	97,94	95,83
Microbispora karnatakensis	96,31	96,07		88,19	95,59	93,41	92,67	89,63	90,27	95,35	95,35	98,41
Microbispora parva	87,26	86,73	88,19		88,58	87,38	86,31	89,5	86,86	88,58	87	87,93
Microbispora thermodiastatica	93,66	93,53	95,59	88,58		94,26	94,74	89,11	90,91	98,75	93,9	95,59
Microbispora thermorosea	92,79	92,17	93,41	87,38	94,26		94,02	86,72	89,89	94,14	93,53	93,41
Microbispora amethystogenes	92,42	92,42	92,67	86,31	94,74	94,02		87,92	89,5	95,1	93,41	93,41
Microbispora corallina	89,11	89,1	89,63	89,5	89,11	86,72	87,92		89,76	88,85	89,37	89,62
Microbispora mesophila	89,89	89,63	90,27	86,86	90,91	89,89	89,5	89,76		90,27	90,02	90,53
Microbispora aerata	93,16	93,28	95,35	88,58	98,75	94,14	95,1	88,85	90,27		93,65	95,59
Microbispora rosea	94,87	97,94	95,35	87	93,9	93,53	93,41	89,37	90,02	93,65		95,59
Microbispora siamensis	96,07	95,83	98,41	87,93	95,59	93,41	93,41	89,62	90,53	95,59	95,59	
LGMB250	92,67	92,42	92,92	86,58	94,98	94,26	99,77	88,19	89,76	95,34	93,41	93,41
LGMB251	94,02	92,55	94,51	88,59	93,54	91,16	91,29	90,27	89,76	92,92	92,05	94,02
LGMB252	95,47	94,75	96,19	87,4	94,63	93,04	93,54	88,45	89,37	94,63	94,75	97,36
LGMB253	95,83	94,99	97,13	87,27	94,27	92,92	92,67	88,58	89,24	94,02	94,63	96,78
LGMB255	92,67	92,42	92,79	86,58	94,86	94,14	99,66	88,19	89,76	95,22	93,41	93,29
LGMB256	92,67	92,42	92,92	86,58	94,98	94,26	99,77	88,19	89,76	95,34	93,41	93,41
LGMB257	92,67	92,42	92,79	86,58	94,86	94,14	99,66	88,19	89,76	95,22	93,41	93,29
LGMB258	95,47	95,47	95,59	87,4	94,26	93,41	94,27	88,45	89,76	94,51	95,47	96,31
LGMB259	92,92	92,17	93,29	89,11	93,04	89,63	90,53	89,49	89,63	92,3	91,79	93,54
LGMB260a	95,59	94,87	96,54	87,26	94,75	93,41	93,9	88,32	89,5	94,75	95,11	97,48
LGMB261a	95,71	94,75	96,42	87,13	94,63	93,29	93,78	88,19	89,37	94,63	94,99	97,36
Actinomadura echinospora	86,04	86,03	86,18	87,1	86,84	87,1	86,16	86,99	84,94	86,71	86,57	87,39

Table S4. Similarity percentage matrix for the gene *rpo*B (continued)

	LGMB250	LGMB251	LGMB252	LGMB253	LGMB255	LGBM256	LGMB257	LGMB258	LGMB259	LGMB260a	LGMB261a	*Actinomadura echinospora*
Microbispora chromogenes	92,67	94,02	95,47	95,83	92,67	92,67	92,67	95,47	92,92	95,59	95,71	86,04
Microbispora indica	92,42	92,55	94,75	94,99	92,42	92,42	92,42	95,47	92,17	94,87	94,75	86,03
Microbispora karnatakensis	92,92	94,51	96,19	97,13	92,79	92,92	92,79	95,59	93,29	96,54	96,42	86,18
Microbispora parva	86,58	88,59	87,4	87,27	86,58	86,58	86,58	87,4	89,11	87,26	87,13	87,1
Microbispora thermodiastatica	94,98	93,54	94,63	94,27	94,86	94,98	94,86	94,26	93,04	94,75	94,63	86,84
Microbispora thermorosea	94,26	91,16	93,04	92,92	94,14	94,26	94,14	93,41	89,63	93,41	93,29	87,1
Microbispora amethystogenes	99,77	91,29	93,54	92,67	99,66	99,77	99,66	94,27	90,53	93,9	93,78	86,16
Microbispora corallina	88,19	90,27	88,45	88,58	88,19	88,19	88,19	88,45	89,49	88,32	88,19	86,99
Microbispora mesophila	89,76	89,76	89,37	89,24	89,76	89,76	89,76	89,76	89,63	89,5	89,37	84,94
Microbispora aerata	95,34	92,92	94,63	94,02	95,22	95,34	95,22	94,51	92,3	94,75	94,63	86,71
Microbispora rosea	93,41	92,05	94,75	94,63	93,41	93,41	93,41	95,47	91,79	95,11	94,99	86,57
Microbispora siamensis	93,41	94,02	97,36	96,78	93,29	93,41	93,29	96,31	93,54	97,48	97,36	87,39
LGMB250		91,55	93,54	92,92	99,89	100	99,89	94,27	90,53	93,9	93,78	86,16
LGMB251	91,55		94,63	95,71	91,42	91,55	91,42	94,02	95,83	94,75	94,63	85,09
LGMB252	93,54	94,63		98,06	93,41	93,54	93,41	97,36	92,79	99,66	99,55	85,91
LGMB253	92,92	95,71	98,06		92,8	92,92	92,8	96,07	92,67	98,41	98,29	85,36
LGMB255	99,89	91,42	93,41	92,8		99,89	100	94,15	90,4	93,78	93,66	86,03
LGMB256	100	91,55	93,54	92,92	99,89		99,89	94,27	90,53	93,9	93,78	86,16
LGMB257	99,89	91,42	93,41	92,8	100	99,89		94,15	90,4	93,78	93,66	86,03
LGMB258	94,27	94,02	97,36	96,07	94,15	94,27	94,15		92,54	97,71	97,6	85,76
LGMB259	90,53	95,83	92,79	92,67	90,4	90,53	90,4	92,54		92,67	92,79	86,45
LGMB260a	93,9	94,75	99,66	98,41	93,78	93,9	93,78	97,71	92,67		99,89	85,63
LGMB261a	93,78	94,63	99,55	98,29	93,66	93,78	93,66	97,6	92,79	99,89		85,49
Actinomadura echinospora	86,16	85,09	85,91	85,36	86,03	86,16	86,03	85,76	86,45	85,63	85,49	

Table S5. Similarity percentage matrix for the gene 23S rRNA

	Microbispora chromogenes	*Microbispora indica*	*Microbispora karnatakensis*	*Microbispora parva*	*Microbispora thermodiastatica*	*Microbispora thermorosea*	*Microbispora amethystogenes*	*Microbispora corallina*	*Microbispora mesophila*	*Microbispora aerata*	*Microbispora rosea*	*Microbispora siamensis*
Microbispora chromogenes		98,19	99,5	91,53	96,04	95,73	97,58	95,94	95,51	96,04	98,29	99,5
Microbispora indica	98,19		98,5	91,86	95,52	95,2	97,58	96,36	94,77	95,52	99,9	98,5
Microbispora karnatakensis	99,5	98,5		91,86	96,56	96,25	97,48	95,94	95,62	96,56	98,6	100
Microbispora parva	91,53	91,86	91,86		91,86	91,85	91,75	92,42	89,71	91,96	91,97	91,86
Microbispora thermodiastatica	96,04	95,52	96,56	91,86		99,7	94,34	94,03	94,02	99,6	95,62	96,56
Microbispora thermorosea	95,73	95,2	96,25	91,85	99,7		94,02	93,71	93,91	99,7	95,31	96,25
Microbispora amethystogenes	97,58	97,58	97,48	91,75	94,34	94,02		96,04	94,66	94,34	97,68	97,48
Microbispora corallina	95,94	96,36	95,94	92,42	94,03	93,71	96,04		93,7	94,03	96,46	95,94
Microbispora mesophila	95,51	94,77	95,62	89,71	94,02	93,91	94,66	93,7		94,02	94,87	95,62
Microbispora aerata	96,04	95,52	96,56	91,96	99,6	99,7	94,34	94,03	94,02		95,62	96,56
Microbispora rosea	98,29	99,9	98,6	91,97	95,62	95,31	97,68	96,46	94,87	95,62		98,6
Microbispora siamensis	99,5	98,5	100	91,86	96,56	96,25	97,48	95,94	95,62	96,56	98,6	
LGMB250	97,99	97,79	97,89	91,41	94,77	94,45	99,6	96,46	95,08	94,77	97,89	97,89
LGMB251	97,28	97,08	97,39	91,2	94,99	94,89	96,77	95,1	94,35	95,2	96,97	97,39
LGMB252	96,25	95,51	96,35	92,29	97,79	97,69	94,76	93,7	93,05	97,99	95,61	96,35
LGMB253	96,35	95,61	96,45	92,29	97,69	97,59	94,87	93,81	93,16	97,89	95,71	96,45
LGMB255	97,99	97,79	97,89	91,41	94,77	94,45	99,6	96,46	95,08	94,77	97,89	97,89
LGMB256	97,99	97,79	97,89	91,41	94,77	94,45	99,6	96,46	95,08	94,77	97,89	97,89
LGMB257	97,99	97,79	97,89	91,41	94,77	94,45	99,6	96,46	95,08	94,77	97,89	97,89
LGMB258	98	97,59	98,1	91,52	95,73	95,62	96,76	95,1	94,98	95,94	97,48	98,1
LGMB259	98	97,59	98,1	91,52	95,73	95,62	96,76	95,1	94,98	95,94	97,48	98,1
LGMB260a	96,25	95,51	96,35	92,29	97,79	97,69	94,76	93,7	93,05	97,99	95,61	96,35
LGMB261a	96,25	95,51	96,35	92,29	97,79	97,69	94,76	93,7	93,05	97,99	95,61	96,35
Actinomadura echinospora	87,45	86,74	87,45	85,92	87,33	87,21	88,27	87,33	87,32	87,33	86,85	87,45

Table S5. Similarity percentage matrix for the gene 23S rRNA (continued)

	LGMB250	LGMB251	LGMB252	LGMB253	LGMB255	LGMB256	LGMB257	LGMB258	LGMB259	LGMB260a	LGMB261a	*Actinomadura echinospora*
Microbispora chromogenes	97,99	97,28	96,25	96,35	97,99	97,99	97,99	98	98	96,25	96,25	87,45
Microbispora indica	97,79	97,08	95,51	95,61	97,79	97,79	97,79	97,59	97,59	95,51	95,51	86,74
Microbispora karnatakensis	97,89	97,39	96,35	96,45	97,89	97,89	97,89	98,1	98,1	96,35	96,35	87,45
Microbispora parva	91,41	91,2	92,29	92,29	91,41	91,41	91,41	91,52	91,52	92,29	92,29	85,92
Microbispora thermodiastatica	94,77	94,99	97,79	97,69	94,77	94,77	94,77	95,73	95,73	97,79	97,79	87,33
Microbispora thermorosea	94,45	94,89	97,69	97,59	94,45	94,45	94,45	95,62	95,62	97,69	97,69	87,21
Microbispora amethystogenes	99,6	96,77	94,76	94,87	99,6	99,6	99,6	96,76	96,76	94,76	94,76	88,27
Microbispora corallina	96,46	95,1	93,7	93,81	96,46	96,46	96,46	95,1	95,1	93,7	93,7	87,33
Microbispora mesophila	95,08	94,35	93,05	93,16	95,08	95,08	95,08	94,98	94,98	93,05	93,05	87,32
Microbispora aerata	94,77	95,2	97,99	97,89	94,77	94,77	94,77	95,94	95,94	97,99	97,99	87,33
Microbispora rosea	97,89	96,97	95,61	95,71	97,89	97,89	97,89	97,48	97,48	95,61	95,61	86,85
Microbispora siamensis	97,89	97,39	96,35	96,45	97,89	97,89	97,89	98,1	98,1	96,35	96,35	87,45
LGMB250		96,97	94,55	94,65	100	100	100	96,96	96,96	94,55	94,55	88,38
LGMB251	96,97		95,31	95,42	96,97	96,97	96,97	99,31	99,31	95,31	95,31	88,03
LGMB252	94,55	95,31		99,9	94,55	94,55	94,55	96,04	96,04	100	100	86,86
LGMB253	94,65	95,42	99,9		94,65	94,65	94,65	96,15	96,15	99,9	99,9	86,98
LGMB255	100	96,97	94,55	94,65		100	100	96,96	96,96	94,55	94,55	88,38
LGMB256	100	96,97	94,55	94,65	100		100	96,96	96,96	94,55	94,55	88,38
LGMB257	100	96,97	94,55	94,65	100	100		96,96	96,96	94,55	94,55	88,38
LGMB258	96,96	99,31	96,04	96,15	96,96	96,96	96,96		100	96,04	96,04	87,8
LGMB259	96,96	99,31	96,04	96,15	96,96	96,96	96,96	100		96,04	96,04	87,8
LGMB260a	94,55	95,31	100	99,9	94,55	94,55	94,55	96,04	96,04		100	86,86
LGMB261a	94,55	95,31	100	99,9	94,55	94,55	94,55	96,04	96,04	100		86,86
Actinomadura echinospora	88,38	88,03	86,86	86,98	88,38	88,38	88,38	87,8	87,8	86,86	86,86	

Table S6. Similarity percentage matrix for the gene 16S rRNA

	Microbispora chromogenes	*Microbispora indica*	*Microbispora karnatakensis*	*Microbispora parva*	*Microbispora thermodiastatica*	*Microbispora thermorosea*	*Microbispora amethystogenes*	*Microbispora corallina*	*Microbispora mesophila*	*Microbispora aerata*	*Microbispora rosea*	*Microbispora siamensis*
Microbispora chromogenes		97,41	98,4	97,59	97,32	98,32	98,23	97,95	93,01	97,6	96,95	98,22
Microbispora indica	97,41		98,49	99,12	98,32	97,32	97,95	98,04	93,69	98,23	99,56	96,95
Microbispora karnatakensis	98,4	98,49		98,49	98,14	97,5	99,12	98,31	93,69	98,41	98,04	97,86
Microbispora parva	97,59	99,12	98,49		99,03	97,87	98,49	98,23	93,3	98,94	98,67	97,14
Microbispora thermodiastatica	97,32	98,32	98,14	99,03		98,4	98,14	98,05	93,12	99,56	97,96	96,96
Microbispora thermorosea	98,32	97,32	97,5	97,87	98,4		97,14	98,04	92,82	98,49	96,95	97,59
Microbispora amethystogenes	98,23	97,95	99,12	98,49	98,14	97,14		97,87	93,11	98,41	97,5	97,14
Microbispora corallina	97,95	98,04	98,31	98,23	98,05	98,04	97,87		93,39	97,95	97,59	97,77
Microbispora mesophila	93,01	93,69	93,69	93,3	93,12	92,82	93,11	93,39		93,21	93,5	92,32
Microbispora aerata	97,6	98,23	98,41	98,94	99,56	98,49	98,41	97,95	93,21		97,77	97,05
Microbispora rosea	96,95	99,56	98,04	98,67	97,96	96,95	97,5	97,59	93,5	97,77		96,86
Microbispora siamensis	98,22	96,95	97,86	97,14	96,96	97,59	97,14	97,77	92,32	97,05	96,86	
LGMB250	98,4	98,31	99,29	97,95	97,59	96,96	98,76	97,77	93,59	98,05	98,22	97,68
LGMB251	98,49	98,4	99,38	98,04	97,69	97,05	98,85	97,86	93,69	98,14	98,31	97,77
LGMB252	97,87	97,77	98,85	97,5	97,14	96,5	98,32	97,32	93,02	97,6	97,68	97,23
LGMB253	98,32	98,22	99,3	97,96	97,6	96,96	98,67	97,77	93,5	98,05	98,13	97,68
LGMB255	98,32	98,22	99,21	97,86	97,5	96,87	99,03	97,95	93,59	97,78	97,77	97,23
LGMB256	99,12	97,05	98,04	96,87	96,59	97,6	97,87	97,87	92,72	96,87	96,95	98,22
LGMB257	98,76	96,68	97,68	96,5	96,22	97,23	97,5	97,5	92,53	96,5	96,59	97,86
LGMB258	97,14	98,85	98,23	98,67	97,87	97,05	97,5	97,59	93,31	98,14	98,76	97,05
LGMB259	97,41	99,12	98,49	98,94	98,14	97,32	97,77	97,86	93,59	98,41	99,03	97,32
LGMB260a	98,49	98,4	99,38	98,04	97,69	97,05	98,85	97,86	93,69	98,14	98,31	97,77
LGMB261a	98,41	98,31	99,29	97,95	97,59	96,96	98,76	97,77	93,59	98,05	98,22	97,68
Actinomadura echinospora	93,19	93,1	93,19	93,48	93,48	92,71	93,58	93,09	92,72	93,39	93,1	92,5

Table S6. Similarity percentage matrix for the gene 16S rRNA (continued)

	LGMB250	LGMB251	LGMB252	LGMB253	LGMB255	LGMB256	LGMB257	LGMB258	LGMB259	LGMB260a	LGMB261a	*Actinomadura echinospora*
Microbispora chromogenes	98,4	98,49	97,87	98,32	98,32	99,12	98,76	97,14	97,41	98,49	98,41	93,19
Microbispora indica	98,31	98,4	97,77	98,22	98,22	97,05	96,68	98,85	99,12	98,4	98,31	93,1
Microbispora karnatakensis	99,29	99,38	98,85	99,3	99,21	98,04	97,68	98,23	98,49	99,38	99,29	93,19
Microbispora parva	97,95	98,04	97,5	97,96	97,86	96,87	96,5	98,67	98,94	98,04	97,95	93,48
Microbispora thermodiastatica	97,59	97,69	97,14	97,6	97,5	96,59	96,22	97,87	98,14	97,69	97,59	93,48
Microbispora thermorosea	96,96	97,05	96,5	96,96	96,87	97,6	97,23	97,05	97,32	97,05	96,96	92,71
Microbispora amethystogenes	98,76	98,85	98,32	98,67	99,03	97,87	97,5	97,5	97,77	98,85	98,76	93,58
Microbispora corallina	97,77	97,86	97,32	97,77	97,95	97,87	97,5	97,59	97,86	97,86	97,77	93,09
Microbispora mesophila	93,59	93,69	93,02	93,5	93,59	92,72	92,53	93,31	93,59	93,69	93,59	92,72
Microbispora aerata	98,05	98,14	97,6	98,05	97,78	96,87	96,5	98,14	98,41	98,14	98,05	93,39
Microbispora rosea	98,22	98,31	97,68	98,13	97,77	96,95	96,59	98,76	99,03	98,31	98,22	93,1
Microbispora siamensis	97,68	97,77	97,23	97,68	97,23	98,22	97,86	97,05	97,32	97,77	97,68	92,5
LGMB250		99,91	99,3	99,74	99,21	98,4	98,05	98,58	98,85	99,91	99,82	93,58
LGMB251	99,91		99,38	99,82	99,3	98,49	98,14	98,67	98,94	100	99,91	93,58
LGMB252	99,3	99,38		99,56	98,67	97,87	97,5	98,14	98,41	99,38	99,3	92,91
LGMB253	99,74	99,82	99,56		99,12	98,32	97,96	98,58	98,85	99,82	99,74	93,39
LGMB255	99,21	99,3	98,67	99,12		98,32	97,96	97,96	98,22	99,3	99,21	93,3
LGMB256	98,4	98,49	97,87	98,32	98,32		99,65	97,68	97,41	98,49	98,41	93,01
LGMB257	98,05	98,14	97,5	97,96	97,96	99,65		97,32	97,05	98,14	98,05	92,81
LGMB258	98,58	98,67	98,14	98,58	97,96	97,68	97,32		99,56	98,67	98,58	93,1
LGMB259	98,85	98,94	98,41	98,85	98,22	97,41	97,05	99,56		98,94	98,85	93,39
LGMB260a	99,91	100	99,38	99,82	99,3	98,49	98,14	98,67	98,94		99,91	93,58
LGMB261a	99,82	99,91	99,3	99,74	99,21	98,41	98,05	98,58	98,85	99,91		93,49
Actinomadura echinospora	93,58	93,58	92,91	93,39	93,3	93,01	92,81	93,1	93,39	93,58	93,49	

Table S7. Distance Matrix calculated by using the K2P substitution for the gene 16S rRNA, 23S rRNA, *gyr*B and *rpo*B

	Microbispora chromogenes	***Microbispora indica***	***Microbispora karnatakensis***	***Microbispora parva***	***Microbispora thermodiastatica***	***Microbispora thermorosea***	***Microbispora amethystogenes***	***Microbispora corallina***	***Microbispora mesophila***	***Microbispora aerata***	***Microbispora rosea***	***Microbispora siamensis***
Microbispora chromogenes		0.045162	0.036415	0.060668	0.056023	0.056023	0.050288	0.061805	0.082119	0.054011	0.046869	0.037816
Microbispora indica	0.045162		0.025276	0.077641	0.042045	0.050297	0.041479	0.052289	0.074119	0.040634	0.008331	0.030834
Microbispora karnatakensis	0.036415	0.025276		0.076161	0.033334	0.042615	0.037255	0.050858	0.069712	0.031659	0.026387	0.012669
Microbispora parva	0.060668	0.077641	0.076161		0.073798	0.080314	0.078828	0.069681	0.105452	0.071443	0.077049	0.081509
Microbispora thermodiastatica	0.056023	0.042045	0.033334	0.073798		0.026104	0.045444	0.058909	0.076183	0.009412	0.040352	0.037262
Microbispora thermorosea	0.056023	0.050297	0.042615	0.080314	0.026104		0.050862	0.065005	0.079759	0.025551	0.046597	0.042622
Microbispora amethystogenes	0.050288	0.041479	0.037255	0.078828	0.045444	0.050862		0.055737	0.074707	0.041762	0.038944	0.042338
Microbispora corallina	0.061805	0.052289	0.050858	0.069681	0.058909	0.065005	0.055737		0.078267	0.057181	0.05143	0.051719
Microbispora mesophila	0.082119	0.074119	0.069712	0.105452	0.076183	0.079759	0.074707	0.078267		0.075888	0.072647	0.072981
Microbispora aerata	0.054011	0.040634	0.031659	0.071443	0.009412	0.025551	0.041762	0.057181	0.075888		0.039507	0.035581
Microbispora rosea	0.046869	0.008331	0.026387	0.077049	0.040352	0.046597	0.038944	0.05143	0.072647	0.039507		0.029728
Microbispora siamensis	0.037816	0.030834	0.012669	0.081509	0.037262	0.042622	0.042338	0.051719	0.072981	0.035581	0.029728	
LGMB250	0.048005	0.039223	0.034453	0.08091	0.044877	0.049723	0.006981	0.054585	0.071468	0.040632	0.036134	0.038956
LGMB251	0.048007	0.04233	0.033614	0.078246	0.049431	0.056324	0.034739	0.054585	0.07414	0.046866	0.042895	0.040362
LGMB252	0.048005	0.041772	0.03166	0.078542	0.038942	0.045734	0.036711	0.063551	0.07916	0.035011	0.040362	0.034742
LGMB253	0.046297	0.038114	0.028048	0.078241	0.038942	0.044601	0.038958	0.061228	0.077971	0.035571	0.037553	0.034187
LGMB255	0.048861	0.040069	0.036135	0.081804	0.046582	0.05144	0.007522	0.054585	0.071466	0.042893	0.038101	0.041212
LGMB256	0.046296	0.043461	0.038945	0.085089	0.048574	0.048292	0.009955	0.054873	0.074424	0.044876	0.040355	0.037825
LGMB257	0.047434	0.044878	0.040919	0.086288	0.050574	0.050291	0.012127	0.056024	0.074718	0.046866	0.041767	0.039232
LGMB258	0.044876	0.030823	0.030266	0.076162	0.041197	0.047439	0.032227	0.05862	0.072936	0.037816	0.029988	0.032778
LGMB259	0.052293	0.039788	0.037536	0.07351	0.047434	0.057182	0.04008	0.0566	0.073243	0.045728	0.040069	0.041208
LGMB260a	0.045729	0.039515	0.029156	0.077059	0.036974	0.043184	0.034191	0.0621	0.076787	0.033054	0.037547	0.032789
LGMB261a	0.045729	0.040077	0.029711	0.07765	0.037536	0.043749	0.034749	0.06268	0.077377	0.033612	0.038108	0.033346
Actinomadura echinospora	0.124108	0.127579	0.125995	0.125366	0.123471	0.126628	0.122535	0.123472	0.129164	0.122212	0.124734	0.126319

Table S7. Distance Matrix calculated by using the K2P substitution for the gene 16S rRNA, 23S rRNA, *gyr*B and *rpo*B (continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGMB256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora chromogenes	0.048005	0.048007	0.048005	0.046297	0.048861	0.046296	0.047434	0.044876	0.052293	0.045729	0.045729	0.124108
Microbispora indica	0.039223	0.04233	0.041772	0.038114	0.040069	0.043461	0.044878	0.030823	0.039788	0.039515	0.040077	0.127579
Microbispora karnatakensis	0.034453	0.033614	0.03166	0.028048	0.036135	0.038945	0.040919	0.030266	0.037536	0.029156	0.029711	0.125995
Microbispora parva	0.08091	0.078246	0.078542	0.078241	0.081804	0.085089	0.086288	0.076162	0.07351	0.077059	0.07765	0.125366
Microbispora thermodiastatica	0.044877	0.049431	0.038942	0.038942	0.046582	0.048574	0.050574	0.041197	0.047434	0.036974	0.037536	0.123471
Microbispora thermorosea	0.049723	0.056324	0.045734	0.044601	0.05144	0.048292	0.050291	0.047439	0.057182	0.043184	0.043749	0.126628
Microbispora amethystogenes	0.006981	0.034739	0.036711	0.038958	0.007522	0.009955	0.012127	0.032227	0.04008	0.034191	0.034749	0.122535
Microbispora corallina	0.054585	0.054585	0.063551	0.061228	0.054585	0.054873	0.056024	0.05862	0.0566	0.0621	0.06268	0.123472
Microbispora mesophila	0.071468	0.07414	0.07916	0.077971	0.071466	0.074424	0.074718	0.072936	0.073243	0.076787	0.077377	0.129164
Microbispora aerata	0.040632	0.046866	0.035011	0.035571	0.042893	0.044876	0.046866	0.037816	0.045728	0.033054	0.033612	0.122212
Microbispora rosea	0.036134	0.042895	0.040362	0.037553	0.038101	0.040355	0.041767	0.029988	0.040069	0.037547	0.038108	0.124734
Microbispora siamensis	0.038956	0.040362	0.034742	0.034187	0.041212	0.037825	0.039232	0.032778	0.041208	0.032789	0.033346	0.126319
LGMB250		0.030831	0.033627	0.035025	0.00429	0.005365	0.007793	0.027776	0.036705	0.030838	0.031394	0.123166
LGMB251	0.030831		0.030827	0.028323	0.034189	0.035587	0.037838	0.023618	0.015117	0.028602	0.029157	0.126944
LGMB252	0.033627	0.030827		0.00806	0.036435	0.038403	0.040095	0.025828	0.036416	0.002677	0.003214	0.129482
LGMB253	0.035025	0.028323	0.00806		0.037838	0.039809	0.041504	0.028877	0.036694	0.006442	0.006981	0.127896
LGMB255	0.00429	0.034189	0.036435	0.037838		0.006713	0.006173	0.031676	0.04009	0.033636	0.034193	0.125056
LGMB256	0.005365	0.035587	0.038403	0.039809	0.006713		0.002678	0.03112	0.0415	0.035596	0.036155	0.125371
LGMB257	0.007793	0.037838	0.040095	0.041504	0.006173	0.002678		0.033638	0.043768	0.037282	0.037842	0.126633
LGMB258	0.027776	0.023618	0.025828	0.028877	0.031676	0.03112	0.033638		0.022518	0.023343	0.023895	0.126314
LGMB259	0.036705	0.015117	0.036416	0.036694	0.04009	0.0415	0.043768	0.022518		0.035014	0.035014	0.125363
LGMB260a	0.030838	0.028602	0.002677	0.006442	0.033636	0.035596	0.037282	0.023343	0.035014		0.000535	0.127896
LGMB261a	0.031394	0.029157	0.003214	0.006981	0.034193	0.036155	0.037842	0.023895	0.035014	0.000535		0.128531
Actinomadura echinospora	0.123166	0.126944	0.129482	0.127896	0.125056	0.125371	0.126633	0.126314	0.125363	0.127896	0.128531	

Table S8. Distance Matrix calculated by using the K2P substitution for the gene *gyr*B and *rpo*B

	Microbispora chromogenes	***Microbispora indica***	***Microbispora karnatakensis***	***Microbispora parva***	***Microbispora thermodiastatica***	***Microbispora thermorosea***	***Microbispora amethystogenes***	***Microbispora corallina***	***Microbispora mesophila***	***Microbispora aerata***	***Microbispora rosea***	***Microbispora siamensis***
Microbispora chromogenes		0.077605	0.072758	0.072681	0.088899	0.094558	0.092393	0.107519	0.116276	0.086064	0.079001	0.074806
Microbispora indica	0.077605		0.039461	0.128927	0.058966	0.069232	0.068596	0.087464	0.096667	0.054883	0.015912	0.041435
Microbispora karnatakensis	0.072758	0.039461		0.119985	0.043425	0.058931	0.066495	0.083211	0.091652	0.041426	0.039445	0.014625
Microbispora parva	0.072681	0.128927	0.119985		0.118601	0.12527	0.125989	0.105333	0.136406	0.112697	0.124442	0.122187
Microbispora thermodiastatica	0.088899	0.058966	0.043425	0.118601		0.048794	0.058336	0.088165	0.092381	0.016555	0.052853	0.044093
Microbispora thermorosea	0.094558	0.069232	0.058931	0.12527	0.048794		0.061714	0.101025	0.098095	0.04813	0.058255	0.059615
Microbispora amethystogenes	0.092393	0.068596	0.066495	0.125989	0.058336	0.061714		0.092438	0.093096	0.051556	0.059657	0.063721
Microbispora corallina	0.107519	0.087464	0.083211	0.105333	0.088165	0.101025	0.092438		0.097386	0.083177	0.082505	0.081087
Microbispora mesophila	0.116276	0.096667	0.091652	0.136406	0.092381	0.098095	0.093096	0.097386		0.092376	0.092376	0.089519
Microbispora aerata	0.086064	0.054883	0.041426	0.112697	0.016555	0.04813	0.051556	0.083177	0.092376		0.049484	0.040763
Microbispora rosea	0.079001	0.015912	0.039445	0.124442	0.052853	0.058255	0.059657	0.082505	0.092376	0.049484		0.038771
Microbispora siamensis	0.074806	0.041435	0.014625	0.122187	0.044093	0.059615	0.063721	0.081087	0.089519	0.040763	0.038771	
LGMB250	0.090973	0.06722	0.063745	0.124506	0.055614	0.060334	0.005055	0.091722	0.091673	0.048859	0.059666	0.062352
LGMB251	0.086687	0.070618	0.058936	0.116986	0.068537	0.07965	0.053502	0.083195	0.09379	0.067153	0.070623	0.063029
LGMB252	0.074792	0.054203	0.043443	0.120701	0.057588	0.068531	0.041426	0.091745	0.092372	0.052834	0.050828	0.038774
LGMB253	0.074792	0.049464	0.038774	0.123677	0.06031	0.068529	0.050128	0.090315	0.093801	0.056899	0.048121	0.041426
LGMB255	0.092387	0.068586	0.067173	0.125975	0.058999	0.063745	0.008235	0.093154	0.091673	0.052214	0.06102	0.064408
LGMB256	0.092393	0.067894	0.065116	0.125989	0.056968	0.061698	0.00569	0.093143	0.092376	0.050201	0.060334	0.063721
LGMB257	0.092387	0.068586	0.067173	0.125975	0.058999	0.063745	0.008235	0.093154	0.091673	0.052214	0.06102	0.064408
LGMB258	0.073418	0.049506	0.046809	0.119243	0.054897	0.063038	0.037447	0.091007	0.092376	0.050166	0.046137	0.044103
LGMB259	0.093801	0.073404	0.066483	0.114796	0.072025	0.088825	0.058258	0.088175	0.095221	0.071317	0.072721	0.066463
LGMB260a	0.074101	0.053531	0.041443	0.121452	0.056912	0.066464	0.039432	0.092449	0.091663	0.052161	0.048809	0.038108
LGMB261a	0.073404	0.05421	0.042111	0.122196	0.057594	0.067153	0.040096	0.093165	0.092376	0.052837	0.049484	0.038771
Actinomadura echinospora	0.16662	0.169946	0.171547	0.162076	0.16681	0.167695	0.171699	0.163559	0.174625	0.162841	0.163607	0.166707

Table S8. Distance Matrix calculated by using the K2P substitution for the gene *gyr*B and *rpo*B (continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGMB256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora chromogenes	0.090973	0.086687	0.074792	0.074792	0.092387	0.092393	0.092387	0.073418	0.093801	0.074101	0.073404	0.16662
Microbispora indica	0.06722	0.070618	0.054203	0.049464	0.068586	0.067894	0.068586	0.049506	0.073404	0.053531	0.05421	0.169946
Microbispora karnatakensis	0.063745	0.058936	0.043443	0.038774	0.067173	0.065116	0.067173	0.046809	0.066483	0.041443	0.042111	0.171547
Microbispora parva	0.124506	0.116986	0.120701	0.123677	0.125975	0.125989	0.125975	0.119243	0.114796	0.121452	0.122196	0.162076
Microbispora thermodiastatica	0.055614	0.068537	0.057588	0.06031	0.058999	0.056968	0.058999	0.054897	0.072025	0.056912	0.057594	0.16681
Microbispora thermorosea	0.060334	0.07965	0.068531	0.068529	0.063745	0.061698	0.063745	0.063038	0.088825	0.066464	0.067153	0.167695
Microbispora amethystogenes	0.005055	0.053502	0.041426	0.050128	0.008235	0.00569	0.008235	0.037447	0.058258	0.039432	0.040096	0.171699
Microbispora corallina	0.091722	0.083195	0.091745	0.090315	0.093154	0.093143	0.093154	0.091007	0.088175	0.092449	0.093165	0.163559
Microbispora mesophila	0.091673	0.09379	0.092372	0.093801	0.091673	0.092376	0.091673	0.092376	0.095221	0.091663	0.092376	0.174625
Microbispora aerata	0.048859	0.067153	0.052834	0.056899	0.052214	0.050201	0.052214	0.050166	0.071317	0.052161	0.052837	0.162841
Microbispora rosea	0.059666	0.070623	0.050828	0.048121	0.06102	0.060334	0.06102	0.046137	0.072721	0.048809	0.049484	0.163607
Microbispora siamensis	0.062352	0.063029	0.038774	0.041426	0.064408	0.063721	0.064408	0.044103	0.066463	0.038108	0.038771	0.166707
LGMB250		0.053503	0.040098	0.047443	0.004423	0.001261	0.004423	0.036125	0.059619	0.038108	0.038771	0.174113
LGMB251	0.053503		0.03877	0.036782	0.056902	0.054184	0.056902	0.042092	0.023682	0.038106	0.038769	0.181056
LGMB252	0.040098	0.03877		0.015269	0.042091	0.040761	0.042091	0.022375	0.049456	0.001892	0.002523	0.173926
LGMB253	0.047443	0.036782	0.015269		0.049455	0.048111	0.049455	0.033488	0.054189	0.013343	0.013985	0.174703
LGMB255	0.004423	0.056902	0.042091	0.049455		0.003791	0	0.040761	0.06303	0.040096	0.040761	0.176435
LGMB256	0.001261	0.054184	0.040761	0.048111	0.003791		0.003791	0.037447	0.060297	0.038769	0.039432	0.174853
LGMB257	0.004423	0.056902	0.042091	0.049455	0	0.003791		0.040761	0.06303	0.040096	0.040761	0.176435
LGMB258	0.036125	0.042092	0.022375	0.033488	0.040761	0.037447	0.040761		0.050813	0.020431	0.021079	0.174015
LGMB259	0.059619	0.023682	0.049456	0.054189	0.06303	0.060297	0.06303	0.050813		0.050132	0.049458	0.173899
LGMB260a	0.038108	0.038106	0.001892	0.013343	0.040096	0.038769	0.040096	0.020431	0.050132		0.00063	0.175536
LGMB261a	0.038771	0.038769	0.002523	0.013985	0.040761	0.039432	0.040761	0.021079	0.049458	0.00063		0.176343
Actinomadura echinospora	0.174113	0.181056	0.173926	0.174703	0.176435	0.174853	0.176435	0.174015	0.173899	0.175536	0.176343	

Table S9. Distance Matrix calculated by using the K2P substitution for the gene *gyr*B

	Microbispora chromogenes	***Microbispora indica***	***Microbispora karnatakensis***	***Microbispora parva***	***Microbispora thermodiastatica***	***Microbispora thermorosea***	***Microbispora amethystogenes***	***Microbispora corallina***	***Microbispora mesophila***	***Microbispora aerata***	***Microbispora rosea***	***Microbispora siamensis***
Microbispora chromogenes		0.122586	0.120889	0.008622	0.122539	0.124194	0.114015	0.105779	0.13594	0.109212	0.115824	0.122425
Microbispora indica	0.122586		0.039641	0.124287	0.051768	0.057894	0.059509	0.061043	0.087812	0.039605	0.01007	0.041109
Microbispora karnatakensis	0.120889	0.039641		0.122586	0.042618	0.050216	0.057957	0.057957	0.084597	0.035096	0.030631	0.01297
Microbispora parva	0.008622	0.124287	0.122586		0.124238	0.124155	0.112346	0.105753	0.14279	0.110882	0.117508	0.124119
Microbispora thermodiastatica	0.122539	0.051768	0.042618	0.124238		0.038097	0.065688	0.062602	0.094254	0.021742	0.042622	0.044146
Microbispora thermorosea	0.124194	0.057894	0.050216	0.124155	0.038097		0.064111	0.062565	0.094254	0.035096	0.050216	0.051778
Microbispora amethystogenes	0.114015	0.059509	0.057957	0.112346	0.065688	0.064111		0.057957	0.078231	0.054846	0.051801	0.06099
Microbispora corallina	0.105779	0.061043	0.057957	0.105753	0.062602	0.062565	0.057957		0.091091	0.048733	0.053321	0.053293
Microbispora mesophila	0.13594	0.087812	0.084597	0.14279	0.094254	0.094254	0.078231	0.091091		0.086199	0.08301	0.083004
Microbispora aerata	0.109212	0.039605	0.035096	0.110882	0.021742	0.035096	0.054846	0.048733	0.086199		0.032109	0.036605
Microbispora rosea	0.115824	0.01007	0.030631	0.117508	0.042622	0.050216	0.051801	0.053321	0.08301	0.032109		0.032107
Microbispora siamensis	0.122425	0.041109	0.01297	0.124119	0.044146	0.051778	0.06099	0.053293	0.083004	0.036605	0.032107	
LGMB250	0.114041	0.05643	0.054884	0.11237	0.062582	0.064101	0.008622	0.059509	0.078237	0.051783	0.051823	0.057899
LGMB251	0.12235	0.065673	0.064143	0.120664	0.073506	0.068794	0.012975	0.065688	0.083	0.062553	0.059471	0.067211
LGMB252	0.114041	0.05643	0.050249	0.114071	0.062582	0.067226	0.01297	0.062625	0.075068	0.051783	0.048753	0.054815
LGMB253	0.119078	0.048733	0.051783	0.119113	0.064143	0.065661	0.021741	0.061066	0.076651	0.053321	0.041138	0.053279
LGMB255	0.117341	0.059488	0.061043	0.115665	0.068808	0.070346	0.014445	0.062625	0.078237	0.057921	0.054863	0.060998
LGMB256	0.117366	0.057937	0.057957	0.115689	0.065688	0.067216	0.010078	0.062602	0.079815	0.054846	0.053321	0.06099
LGMB257	0.117341	0.059488	0.061043	0.115665	0.068808	0.070346	0.014445	0.062625	0.078237	0.057921	0.054863	0.060998
LGMB258	0.110762	0.054908	0.050286	0.110731	0.051768	0.059447	0.01297	0.061092	0.07984	0.044158	0.047244	0.053284
LGMB259	0.12404	0.067239	0.065707	0.12235	0.075088	0.070366	0.014428	0.067255	0.084597	0.064111	0.061024	0.068777
LGMB260a	0.114041	0.05643	0.050249	0.114071	0.062582	0.067226	0.01297	0.062625	0.075068	0.051783	0.048753	0.054815
LGMB261a	0.114041	0.05643	0.050249	0.114071	0.062582	0.067226	0.01297	0.062625	0.075068	0.051783	0.048753	0.054815
Actinomadura echinospora	0.202483	0.210267	0.216257	0.206215	0.214033	0.219947	0.216257	0.208529	0.206357	0.202793	0.202627	0.22171

Table S9. Distance Matrix calculated by using the K2P substitution for the gene *gyr*B (continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGMB256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora chromogenes	0.114041	0.12235	0.114041	0.119078	0.117341	0.117366	0.117341	0.110762	0.12404	0.114041	0.114041	0.202483
Microbispora indica	0.05643	0.065673	0.05643	0.048733	0.059488	0.057937	0.059488	0.054908	0.067239	0.05643	0.05643	0.210267
Microbispora karnatakensis	0.054884	0.064143	0.050249	0.051783	0.061043	0.057957	0.061043	0.050286	0.065707	0.050249	0.050249	0.216257
Microbispora parva	0.11237	0.120664	0.114071	0.119113	0.115665	0.115689	0.115665	0.110731	0.12235	0.114071	0.114071	0.206215
Microbispora thermodiastatica	0.062582	0.073506	0.062582	0.064143	0.068808	0.065688	0.068808	0.051768	0.075088	0.062582	0.062582	0.214033
Microbispora thermorosea	0.064101	0.068794	0.067226	0.065661	0.070346	0.067216	0.070346	0.059447	0.070366	0.067226	0.067226	0.219947
Microbispora amethystogenes	0.008622	0.012975	0.01297	0.021741	0.014445	0.010078	0.014445	0.01297	0.014428	0.01297	0.01297	0.216257
Microbispora corallina	0.059509	0.065688	0.062625	0.061066	0.062625	0.062602	0.062625	0.061092	0.067255	0.062625	0.062625	0.208529
Microbispora mesophila	0.078237	0.083	0.075068	0.076651	0.078237	0.079815	0.078237	0.07984	0.084597	0.075068	0.075068	0.206357
Microbispora aerata	0.051783	0.062553	0.051783	0.053321	0.057921	0.054846	0.057921	0.044158	0.064111	0.051783	0.051783	0.202793
Microbispora rosea	0.051823	0.059471	0.048753	0.041138	0.054863	0.053321	0.054863	0.047244	0.061024	0.048753	0.048753	0.202627
Microbispora siamensis	0.057899	0.067211	0.054815	0.053279	0.060998	0.06099	0.060998	0.053284	0.068777	0.054815	0.054815	0.22171
LGMB250		0.01589	0.01007	0.018814	0.008627	0.002863	0.008627	0.01007	0.017348	0.01007	0.01007	0.222115
LGMB251	0.01589		0.02028	0.029133	0.021781	0.017367	0.021781	0.020272	0.00143	0.02028	0.02028	0.22386
LGMB252	0.01007	0.02028		0.010076	0.012969	0.011517	0.012969	0.017348	0.021747	0	0	0.218203
LGMB253	0.018814	0.029133	0.010076		0.021742	0.020272	0.021742	0.02618	0.030618	0.010076	0.010076	0.212381
LGMB255	0.008627	0.021781	0.012969	0.021742		0.007186	0	0.018806	0.023248	0.012969	0.012969	0.225825
LGMB256	0.002863	0.017367	0.011517	0.020272	0.007186		0.007186	0.01297	0.018827	0.011517	0.011517	0.22386
LGMB257	0.008627	0.021781	0.012969	0.021742	0	0.007186		0.018806	0.023248	0.012969	0.012969	0.225825
LGMB258	0.01007	0.020272	0.017348	0.02618	0.018806	0.01297	0.018806		0.021741	0.017348	0.017348	0.216365
LGMB259	0.017348	0.00143	0.021747	0.030618	0.023248	0.018827	0.023248	0.021741		0.021747	0.021747	0.225825
LGMB260a	0.01007	0.02028	0	0.010076	0.012969	0.011517	0.012969	0.017348	0.021747		0	0.218203
LGMB261a	0.01007	0.02028	0	0.010076	0.012969	0.011517	0.012969	0.017348	0.021747	0		0.218203
Actinomadura echinospora	0.222115	0.22386	0.218203	0.212381	0.225825	0.22386	0.225825	0.216365	0.225825	0.218203	0.218203	

Table S10. Distance Matrix calculated by using the K2P substitution for the gene *rpo*B

	Microbispora chromogenes	***Microbispora indica***	***Microbispora karnatakensis***	***Microbispora parva***	***Microbispora thermodiastatica***	***Microbispora thermorosea***	***Microbispora amethystogenes***	***Microbispora corallina***	***Microbispora mesophila***	***Microbispora aerata***	***Microbispora rosea***	***Microbispora siamensis***
Microbispora chromogenes		0.044063	0.036945	0.12735	0.063446	0.072052	0.075787	0.108907	0.101134	0.068358	0.051265	0.039312
Microbispora indica	0.044063		0.03932	0.132703	0.06469	0.078299	0.075837	0.108968	0.103724	0.06716	0.02055	0.041694
Microbispora karnatakensis	0.036945	0.03932		0.118057	0.044063	0.065874	0.073302	0.103737	0.09726	0.046455	0.046466	0.015937
Microbispora parva	0.12735	0.132703	0.118057		0.114203	0.126151	0.136941	0.105011	0.131416	0.114165	0.130022	0.120703
Microbispora thermodiastatica	0.063446	0.06469	0.044063	0.114203		0.057375	0.052603	0.108949	0.090924	0.012491	0.061032	0.044065
Microbispora thermorosea	0.072052	0.078299	0.065874	0.126151	0.057375		0.059842	0.132809	0.101146	0.058616	0.064657	0.065876
Microbispora amethystogenes	0.075787	0.075837	0.073302	0.136941	0.052603	0.059842		0.120782	0.105036	0.048987	0.06591	0.065881
Microbispora corallina	0.108907	0.108968	0.103737	0.105011	0.108949	0.132809	0.120782		0.102406	0.111499	0.106338	0.103752
Microbispora mesophila	0.101134	0.103724	0.09726	0.131416	0.090924	0.101146	0.105036	0.102406		0.097289	0.099844	0.094701
Microbispora aerata	0.068358	0.06716	0.046455	0.114165	0.012491	0.058616	0.048987	0.111499	0.097289		0.063491	0.044062
Microbispora rosea	0.051265	0.02055	0.046466	0.130022	0.061032	0.064657	0.06591	0.106338	0.099844	0.063491		0.044077
Microbispora siamensis	0.039312	0.041694	0.015937	0.120703	0.044065	0.065876	0.065881	0.103752	0.094701	0.044062	0.044077	
LGMB250	0.073295	0.075837	0.070818	0.13422	0.050174	0.057393	0.002256	0.118127	0.10244	0.04657	0.06591	0.065881
LGMB251	0.059761	0.07454	0.05489	0.114113	0.064647	0.088351	0.087072	0.097289	0.102408	0.070807	0.079534	0.059752
LGMB252	0.045262	0.052473	0.038124	0.12601	0.053684	0.069571	0.064647	0.115523	0.106301	0.053678	0.052479	0.026366
LGMB253	0.041678	0.050057	0.028696	0.127345	0.057317	0.070812	0.07329	0.114203	0.107603	0.059751	0.05368	0.032214
LGMB255	0.073295	0.075837	0.072052	0.13422	0.051365	0.058598	0.003386	0.118127	0.10244	0.047755	0.06591	0.067108
LGMB256	0.073295	0.075837	0.070818	0.13422	0.050174	0.057393	0.002256	0.118127	0.10244	0.04657	0.06591	0.065881
LGMB257	0.073295	0.075837	0.072052	0.13422	0.051365	0.058598	0.003386	0.118127	0.10244	0.047755	0.06591	0.067108
LGMB258	0.045267	0.045284	0.044086	0.126033	0.057375	0.065881	0.057322	0.115467	0.102411	0.054938	0.045275	0.036945
LGMB259	0.070808	0.078299	0.067108	0.108898	0.069622	0.103705	0.094748	0.105093	0.103713	0.077047	0.082072	0.064645
LGMB260a	0.04407	0.05127	0.034578	0.12735	0.052479	0.065873	0.060973	0.116824	0.105011	0.05247	0.048864	0.025195
LGMB261a	0.042875	0.052479	0.03576	0.128689	0.05369	0.067104	0.062197	0.118148	0.106311	0.05368	0.050069	0.026362
Actinomadura echinospora	0.139553	0.139673	0.138242	0.129025	0.131611	0.128982	0.138422	0.130087	0.150562	0.132864	0.134253	0.126124

Table S10. Distance Matrix calculated by using the K2P substitution for the gene *rpo*B (continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGBM256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora chromogenes	0.073295	0.059761	0.045262	0.041678	0.073295	0.073295	0.073295	0.045267	0.070808	0.04407	0.042875	0.139553
Microbispora indica	0.075837	0.07454	0.052473	0.050057	0.075837	0.075837	0.075837	0.045284	0.078299	0.05127	0.052479	0.139673
Microbispora karnatakensis	0.070818	0.05489	0.038124	0.028696	0.072052	0.070818	0.072052	0.044086	0.067108	0.034578	0.03576	0.138242
Microbispora parva	0.13422	0.114113	0.12601	0.127345	0.13422	0.13422	0.13422	0.126033	0.108898	0.12735	0.128689	0.129025
Microbispora thermodiastatica	0.050174	0.064647	0.053684	0.057317	0.051365	0.050174	0.051365	0.057375	0.069622	0.052479	0.05369	0.131611
Microbispora thermorosea	0.057393	0.088351	0.069571	0.070812	0.058598	0.057393	0.058598	0.065881	0.103705	0.065873	0.067104	0.128982
Microbispora amethystogenes	0.002256	0.087072	0.064647	0.07329	0.003386	0.002256	0.003386	0.057322	0.094748	0.060973	0.062197	0.138422
Microbispora corallina	0.118127	0.097289	0.115523	0.114203	0.118127	0.118127	0.118127	0.115467	0.105093	0.116824	0.118148	0.130087
Microbispora mesophila	0.10244	0.102408	0.106301	0.107603	0.10244	0.10244	0.10244	0.102411	0.103713	0.105011	0.106311	0.150562
Microbispora aerata	0.04657	0.070807	0.053678	0.059751	0.047755	0.04657	0.047755	0.054938	0.077047	0.05247	0.05368	0.132864
Microbispora rosea	0.06591	0.079534	0.052479	0.05368	0.06591	0.06591	0.06591	0.045275	0.082072	0.048864	0.050069	0.134253
Microbispora siamensis	0.065881	0.059752	0.026366	0.032214	0.067108	0.065881	0.067108	0.036945	0.064645	0.025195	0.026362	0.126124
LGMB250		0.084546	0.064647	0.070808	0.001127	0	0.001127	0.057322	0.094748	0.060973	0.062197	0.138422
LGMB251	0.084546		0.053678	0.042871	0.085809	0.084546	0.085809	0.059755	0.041716	0.05247	0.05368	0.149119
LGMB252	0.064647	0.053678		0.019393	0.065874	0.064647	0.065874	0.026362	0.072068	0.003386	0.004518	0.140946
LGMB253	0.070808	0.042871	0.019393		0.072046	0.070808	0.072046	0.039302	0.073338	0.015934	0.017085	0.146412
LGMB255	0.001127	0.085809	0.065874	0.072046		0.001127	0	0.058535	0.096017	0.062194	0.063419	0.13975
LGMB256	0	0.084546	0.064647	0.070808	0.001127		0.001127	0.057322	0.094748	0.060973	0.062197	0.138422
LGMB257	0.001127	0.085809	0.065874	0.072046	0	0.001127		0.058535	0.096017	0.062194	0.063419	0.13975
LGMB258	0.057322	0.059755	0.026362	0.039302	0.058535	0.057322	0.058535		0.074571	0.02287	0.024034	0.14238
LGMB259	0.094748	0.041716	0.072068	0.073338	0.096017	0.094748	0.096017	0.074571		0.073324	0.072079	0.13552
LGMB260a	0.060973	0.05247	0.003386	0.015934	0.062194	0.060973	0.062194	0.02287	0.073324		0.001127	0.143689
LGMB261a	0.062197	0.05368	0.004518	0.017085	0.063419	0.062197	0.063419	0.024034	0.072079	0.001127		0.145065
Actinomadura echinospora	0.138422	0.149119	0.140946	0.146412	0.13975	0.138422	0.13975	0.14238	0.13552	0.143689	0.145065	

Table S11. Distance Matrix calculated by using the K2P substitution for the gene 16S rRNA

	Microbispora chromogenes	*Microbispora indica*	*Microbispora karnatakensis*	*Microbispora parva*	*Microbispora thermodiastatica*	*Microbispora thermorosea*	*Microbispora amethystogenes*	*Microbispora corallina*	*Microbispora mesophila*	*Microbispora aerata*	*Microbispora rosea*	*Microbispora siamensis*
Microbispora chromogenes		0.0259	0.015959	0.024075	0.026775	0.016844	0.017748	0.020451	0.069882	0.024047	0.030465	0.017754
Microbispora indica	0.0259		0.015081	0.00882	0.016848	0.026815	0.020451	0.019569	0.063148	0.017748	0.004396	0.030491
Microbispora karnatakensis	0.015959	0.015081		0.015056	0.018635	0.024959	0.008816	0.016859	0.063148	0.015945	0.019579	0.021365
Microbispora parva	0.024075	0.00882	0.015056		0.009706	0.02135	0.015066	0.01774	0.066952	0.010597	0.013272	0.028608
Microbispora thermodiastatica	0.026775	0.016848	0.018635	0.009706		0.015965	0.01864	0.019547	0.068848	0.004401	0.020445	0.03042
Microbispora thermorosea	0.016844	0.026815	0.024959	0.02135	0.015965		0.028595	0.01956	0.071797	0.015066	0.030465	0.024067
Microbispora amethystogenes	0.017748	0.020451	0.008816	0.015066	0.01864	0.028595		0.02134	0.068861	0.015945	0.024978	0.028608
Microbispora corallina	0.020451	0.019569	0.016859	0.01774	0.019547	0.01956	0.02134		0.066069	0.020451	0.024094	0.022283
Microbispora mesophila	0.069882	0.063148	0.063148	0.066952	0.068848	0.071797	0.068861	0.066069		0.06788	0.065047	0.076805
Microbispora aerata	0.024047	0.017748	0.015945	0.010597	0.004401	0.015066	0.015945	0.020451	0.06788		0.022257	0.029504
Microbispora rosea	0.030465	0.004396	0.019579	0.013272	0.020445	0.030465	0.024978	0.024094	0.065047	0.022257		0.031414
Microbispora siamensis	0.017754	0.030491	0.021365	0.028608	0.03042	0.024067	0.028608	0.022283	0.076805	0.029504	0.031414	
LGMB250	0.015954	0.016875	0.007055	0.020458	0.024055	0.030434	0.012375	0.022283	0.064097	0.019534	0.017778	0.023193
LGMB251	0.015056	0.015973	0.006168	0.019553	0.023147	0.029517	0.011483	0.021374	0.063133	0.018634	0.016875	0.022283
LGMB252	0.02134	0.022264	0.011485	0.024964	0.028587	0.035	0.01684	0.026795	0.069832	0.024045	0.023173	0.02771
LGMB253	0.016844	0.017761	0.007048	0.020445	0.024047	0.03042	0.013264	0.022264	0.065032	0.019535	0.018664	0.023173
LGMB255	0.016848	0.017769	0.007939	0.021357	0.024959	0.031345	0.009704	0.020458	0.064082	0.022239	0.022283	0.027731
LGMB256	0.008817	0.029545	0.019553	0.031345	0.034073	0.024047	0.02134	0.021345	0.072774	0.031321	0.030465	0.017754
LGMB257	0.012372	0.033208	0.023165	0.035015	0.037754	0.027678	0.024959	0.024964	0.074735	0.034986	0.034133	0.021357
LGMB258	0.028601	0.011489	0.017748	0.013264	0.021337	0.029517	0.024959	0.024067	0.066938	0.018634	0.012383	0.029534
LGMB259	0.02589	0.008835	0.015073	0.010597	0.018644	0.026804	0.022257	0.021374	0.064097	0.015946	0.009727	0.026826
LGMB260a	0.015056	0.015973	0.006168	0.019553	0.023147	0.029517	0.011483	0.021374	0.063133	0.018634	0.016875	0.022283
LGMB261a	0.015949	0.016866	0.007051	0.020451	0.02405	0.030427	0.012372	0.022273	0.064082	0.019534	0.017769	0.023183
Actinomadura echinospora	0.068087	0.069039	0.068062	0.06524	0.065163	0.07294	0.064216	0.069066	0.072815	0.066136	0.069039	0.074977

Table S11. Distance Matrix calculated by using the K2P substitution for the gene 16S rRNA (Continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGMB256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora Chromogenes	0.015954	0.015056	0.02134	0.016844	0.016848	0.008817	0.012372	0.028601	0.02589	0.015056	0.015949	0.068087
Microbispora indica	0.016875	0.015973	0.022264	0.017761	0.017769	0.029545	0.033208	0.011489	0.008835	0.015973	0.016866	0.069039
Microbispora karnatakensis	0.007055	0.006168	0.011485	0.007048	0.007939	0.019553	0.023165	0.017748	0.015073	0.006168	0.007051	0.068062
Microbispora parva	0.020458	0.019553	0.024964	0.020445	0.021357	0.031345	0.035015	0.013264	0.010597	0.019553	0.020451	0.06524
Microbispora thermodiastatica	0.024055	0.023147	0.028587	0.024047	0.024959	0.034073	0.037754	0.021337	0.018644	0.023147	0.02405	0.065163
Microbispora thermorosea	0.030434	0.029517	0.035	0.03042	0.031345	0.024047	0.027678	0.029517	0.026804	0.029517	0.030427	0.07294
Microbispora amethystogenes	0.012375	0.011483	0.01684	0.013264	0.009704	0.02134	0.024959	0.024959	0.022257	0.011483	0.012372	0.064216
Microbispora corallina	0.022283	0.021374	0.026795	0.022264	0.020458	0.021345	0.024964	0.024067	0.021374	0.021374	0.022273	0.069066
Microbispora mesophila	0.064097	0.063133	0.069832	0.065032	0.064082	0.072774	0.074735	0.066938	0.064097	0.063133	0.064082	0.072815
Microbispora aerata	0.019534	0.018634	0.024045	0.019535	0.022239	0.031321	0.034986	0.018634	0.015946	0.018634	0.019534	0.066136
Microbispora rosea	0.017778	0.016875	0.023173	0.018664	0.022283	0.030465	0.034133	0.012383	0.009727	0.016875	0.017769	0.069039
Microbispora siamensis	0.023193	0.022283	0.02771	0.023173	0.027731	0.017754	0.021357	0.029534	0.026826	0.022283	0.023183	0.074977
LGMB250		0.000877	0.007044	0.002634	0.007935	0.015954	0.019547	0.01416	0.011494	0.000877	0.001755	0.064193
LGMB251	0.000877		0.00616	0.001755	0.007048	0.015056	0.018644	0.013265	0.010601	0	0.000877	0.064193
LGMB252	0.007044	0.00616		0.004395	0.013265	0.02134	0.024959	0.018635	0.015949	0.00616	0.007045	0.0709
LGMB253	0.002634	0.001755	0.004395		0.008817	0.016844	0.02044	0.014156	0.011485	0.001755	0.002634	0.06609
LGMB255	0.007935	0.007048	0.013265	0.008817		0.016848	0.020445	0.020445	0.017761	0.007048	0.007932	0.067041
LGMB256	0.015954	0.015056	0.02134	0.016844	0.016848		0.003514	0.023173	0.02589	0.015056	0.015949	0.069944
LGMB257	0.019547	0.018644	0.024959	0.02044	0.020445	0.003514		0.026804	0.029534	0.018644	0.019542	0.071903
LGMB258	0.01416	0.013265	0.018635	0.014156	0.020445	0.023173	0.026804		0.004395	0.013265	0.014157	0.068968
LGMB259	0.011494	0.010601	0.015949	0.011485	0.017761	0.02589	0.029534	0.004395		0.010601	0.011489	0.066136
LGMB260a	0.000877	0	0.00616	0.001755	0.007048	0.015056	0.018644	0.013265	0.010601		0.000877	0.064193
LGMB261a	0.001755	0.000877	0.007045	0.002634	0.007932	0.015949	0.019542	0.014157	0.011489	0.000877		0.065141
Actinomadura echinospora	0.064193	0.064193	0.0709	0.06609	0.067041	0.069944	0.071903	0.068968	0.066136	0.064193	0.065141	

Table S12. Distance Matrix calculated by using the K2P substitution for the gene 23S rRNA

	Microbispora chromogenes	*Microbispora indica*	*Microbispora karnatakensis*	*Microbispora parva*	*Microbispora thermodiastatica*	*Microbispora thermorosea*	*Microbispora amethystogenes*	*Microbispora corallina*	*Microbispora mesophila*	*Microbispora aerata*	*Microbispora rosea*	*Microbispora siamensis*
Microbispora chromogenes		0.018059	0.00496	0.08473	0.039588	0.042717	0.024168	0.040612	0.044868	0.039588	0.017054	0.00496
Microbispora indica	0.018059		0.015004	0.081374	0.044831	0.047982	0.024168	0.036415	0.052302	0.044831	0.000988	0.015004
Microbispora karnatakensis	0.00496	0.015004		0.081374	0.034363	0.03747	0.0252	0.040612	0.043808	0.034363	0.014002	0
Microbispora parva	0.08473	0.081374	0.081374		0.081447	0.081504	0.082514	0.075847	0.102888	0.080384	0.080282	0.081374
Microbispora thermodiastatica	0.039588	0.044831	0.034363	0.081447		0.002967	0.056593	0.059656	0.059794	0.00396	0.04379	0.034363
Microbispora thermorosea	0.042717	0.047982	0.03747	0.081504	0.002967		0.059794	0.06287	0.060884	0.002967	0.046936	0.03747
Microbispora amethystogenes	0.024168	0.024168	0.0252	0.082514	0.056593	0.059794		0.039552	0.053405	0.056593	0.023155	0.0252
Microbispora corallina	0.040612	0.036415	0.040612	0.075847	0.059656	0.06287	0.039552		0.062956	0.059656	0.035381	0.040612
Microbispora mesophila	0.044868	0.052302	0.043808	0.102888	0.059794	0.060884	0.053405	0.062956		0.059794	0.051252	0.043808
Microbispora aerata	0.039588	0.044831	0.034363	0.080384	0.00396	0.002967	0.056593	0.059656	0.059794		0.04379	0.034363
Microbispora rosea	0.017054	0.000988	0.014002	0.080282	0.04379	0.046936	0.023155	0.035381	0.051252	0.04379		0.014002
Microbispora siamensis	0.00496	0.015004	0	0.081374	0.034363	0.03747	0.0252	0.040612	0.043808	0.034363	0.014002	
LGMB250	0.02009	0.02211	0.021115	0.085881	0.052328	0.055511	0.003959	0.035395	0.049158	0.052328	0.021099	0.021115
LGMB251	0.027167	0.029231	0.026138	0.088007	0.05005	0.051115	0.032329	0.048968	0.056517	0.047951	0.030254	0.026138
LGMB252	0.037536	0.044935	0.036488	0.077123	0.022084	0.023109	0.052355	0.062982	0.069511	0.020061	0.043896	0.036488
LGMB253	0.036504	0.043896	0.035457	0.077094	0.023097	0.024124	0.051306	0.061918	0.068439	0.021072	0.042858	0.035457
LGMB255	0.02009	0.02211	0.021115	0.085881	0.052328	0.055511	0.003959	0.035395	0.049158	0.052328	0.021099	0.021115
LGMB256	0.02009	0.02211	0.021115	0.085881	0.052328	0.055511	0.003959	0.035395	0.049158	0.052328	0.021099	0.021115
LGMB257	0.02009	0.02211	0.021115	0.085881	0.052328	0.055511	0.003959	0.035395	0.049158	0.052328	0.021099	0.021115
LGMB258	0.020039	0.024137	0.019019	0.084756	0.042703	0.043758	0.032414	0.04903	0.050179	0.040625	0.025153	0.019019
LGMB259	0.020039	0.024137	0.019019	0.084756	0.042703	0.043758	0.032414	0.04903	0.050179	0.040625	0.025153	0.019019
LGMB260a	0.037536	0.044935	0.036488	0.077123	0.022084	0.023109	0.052355	0.062982	0.069511	0.020061	0.043896	0.036488
LGMB261a	0.037536	0.044935	0.036488	0.077123	0.022084	0.023109	0.052355	0.062982	0.069511	0.020061	0.043896	0.036488
Actinomadura echinospora	0.125548	0.132633	0.125548	0.140753	0.126669	0.127851	0.11733	0.126669	0.126756	0.126669	0.131465	0.125548

Table S12. Distance Matrix calculated by using the K2P substitution for the gene 23S rRNA (Continued)

	LGMB250	**LGMB251**	**LGMB252**	**LGMB253**	**LGMB255**	**LGMB256**	**LGMB257**	**LGMB258**	**LGMB259**	**LGMB260a**	**LGMB261a**	***Actinomadura echinospora***
Microbispora chromogenes	0.02009	0.027167	0.037536	0.036504	0.02009	0.02009	0.02009	0.020039	0.020039	0.037536	0.037536	0.125548
Microbispora indica	0.02211	0.029231	0.044935	0.043896	0.02211	0.02211	0.02211	0.024137	0.024137	0.044935	0.044935	0.132633
Microbispora karnatakensis	0.021115	0.026138	0.036488	0.035457	0.021115	0.021115	0.021115	0.019019	0.019019	0.036488	0.036488	0.125548
Microbispora parva	0.085881	0.088007	0.077123	0.077094	0.085881	0.085881	0.085881	0.084756	0.084756	0.077123	0.077123	0.140753
Microbispora thermodiastatica	0.052328	0.05005	0.022084	0.023097	0.052328	0.052328	0.052328	0.042703	0.042703	0.022084	0.022084	0.126669
Microbispora thermorosea	0.055511	0.051115	0.023109	0.024124	0.055511	0.055511	0.055511	0.043758	0.043758	0.023109	0.023109	0.127851
Microbispora amethystogenes	0.003959	0.032329	0.052355	0.051306	0.003959	0.003959	0.003959	0.032414	0.032414	0.052355	0.052355	0.11733
Microbispora corallina	0.035395	0.048968	0.062982	0.061918	0.035395	0.035395	0.035395	0.04903	0.04903	0.062982	0.062982	0.126669
Microbispora mesophila	0.049158	0.056517	0.069511	0.068439	0.049158	0.049158	0.049158	0.050179	0.050179	0.069511	0.069511	0.126756
Microbispora aerata	0.052328	0.047951	0.020061	0.021072	0.052328	0.052328	0.052328	0.040625	0.040625	0.020061	0.020061	0.126669
Microbispora rosea	0.021099	0.030254	0.043896	0.042858	0.021099	0.021099	0.021099	0.025153	0.025153	0.043896	0.043896	0.131465
Microbispora siamensis	0.021115	0.026138	0.036488	0.035457	0.021115	0.021115	0.021115	0.019019	0.019019	0.036488	0.036488	0.125548
LGMB250		0.030279	0.054515	0.053464	0	0	0	0.030369	0.030369	0.054515	0.054515	0.116192
LGMB251	0.030279		0.046879	0.045833	0.030279	0.030279	0.030279	0.006942	0.006942	0.046879	0.046879	0.119707
LGMB252	0.054515	0.046879		0.000988	0.054515	0.054515	0.054515	0.039562	0.039562	0	0	0.131354
LGMB253	0.053464	0.045833	0.000988		0.053464	0.053464	0.053464	0.038526	0.038526	0.000988	0.000988	0.130184
LGMB255	0	0.030279	0.054515	0.053464		0	0	0.030369	0.030369	0.054515	0.054515	0.116192
LGMB256	0	0.030279	0.054515	0.053464	0		0	0.030369	0.030369	0.054515	0.054515	0.116192
LGMB257	0	0.030279	0.054515	0.053464	0	0		0.030369	0.030369	0.054515	0.054515	0.116192
LGMB258	0.030369	0.006942	0.039562	0.038526	0.030369	0.030369	0.030369		0	0.039562	0.039562	0.122033
LGMB259	0.030369	0.006942	0.039562	0.038526	0.030369	0.030369	0.030369	0		0.039562	0.039562	0.122033
LGMB260a	0.054515	0.046879	0	0.000988	0.054515	0.054515	0.054515	0.039562	0.039562		0	0.131354
LGMB261a	0.054515	0.046879	0	0.000988	0.054515	0.054515	0.054515	0.039562	0.039562	0		0.131354
Actinomadura echinospora	0.116192	0.119707	0.131354	0.130184	0.116192	0.116192	0.116192	0.122033	0.122033	0.131354	0.131354	

Table S13. Morphologic characteristic of Microbispora sp. on ISP2, ISP3 and ISP4 culture media

Species	Yeast extract-malt extract agar ISP2			Oatmeal agar ISP3			Inorganic salts-starch agar ISP4		
	Aerial spore mass	Substrate mycelium	Grown	Aerial spore mass	Substrate mycelium	Grown	Aerial spore mass	Substrate mycelium	Grown
M. chromogenes	None	With; light pink	+++	Abundant: white	Ivory-white	+++	Ivory-white	Brown/yellow brown	+++
M. indica	None	Ivory -white	+++	Abundant; Ivory-white	Ivory-white	+++	none	Light Brown	+++
M. karnatakensis	None	Light brown	+++	Abundant; white	Ivory-white; and green pigment	+++	none	Brown	+++
M. parva	None	Dark yellow	+++	Abundant; white	Dark pink	+++	none	Dark yellow	+++
M. thermodiastatica	----	-----	-----	none	Ivory-white	+++	------	------	------
M. thermorosea	----	-----	-----	Moderated; white	Ivory-white	+++	none	Light brown	+
M. coralline	None	Red	+++	Abundant; white	Dark Orange; with orange pigment	+++	Moderated; white	Red	++
M. mesophila	None	Light Brown/Dark yellow	+++	Abundant; white	Ivory-white	+++	none	Dark yellow	+++
M. rosea	None	Light orange	+++	Abundant; white	Ivory-white	+++	none	Dark Yellow	++
M. siamensis	None	Light orange	+++	Abundant; white	Ivory-white	+++	none	Dark yellow	+++
LGMB250	None	Brown	++	none	Light pink	+++	none	Brown	+++
LGMB251	None	Ivory-white	+++	none	Ivory-white	+++	none	Ivory-white/light-yellow	+++
LGMB252	None	Ivory-white	+++	Abundant; white	Ivory-white	+++	none	Dark yellow	+
LGMB253	None	Light Brown	+++	Abundant; white	Ivory-white	+++	Moderated; white	Light Brown	+++
LGMB255	None	Light Brown	++	Abundant: white	Ivory-white	+++	none	Light Brown	+++
LGMB256	None	Light Brown	+++	none	Light Brown	+++	none	Light Brown	+++
LGMB257	None	Light Brown	++	Moderated; white	Light Brown	+++	none	Brown	+++
LGMB258	None	Light Brown	++	Abundant; white	Ivory-white	+++	none	Dark yellow	+++
LGMB259	None	Light Orange	+	Moderated; white	Ivory-white	+++	none	Dark yellow	+++
LGMB260a	None	Light Orange	+++	Abundant; white	Ivory-white	+++	none	Light Brown	+++
LGMB261a	None	Light Orange	+++	Abundant; white	Ivory-white	+++	none	Light Brown	+++

+++: Abundant; ++: Moderated; +: low

IX. CONSIDERAÇÕES FINAIS

Actinomicetos endofíticos isolados da planta *Vochysia divergens* demonstraram potencial biotecnológico pela produção de extratos ativos com atividade antioxidante, antitumoral e antimicrobiana. O isolado LGMB259 foi cultivado em meio R5A e produziu sete compostos identificados, quatro β-cabolinas e três indóis, sendo que o metabólito 1-vinil-*β*-carbolina-3-ácido carboxílico apresentou atividade antibacteriana, antifúngica e citotóxica. O presente trabalho também trouxe novos "insights" em relação à estrutura-atividade dentro do grupo *β*-carbolina, e mostrou que o grupamento vinil é essencial para a atividade biológica observada.

Através de análises multigênicas (MLSA) para o gênero *Microbispora* sugere-se que sequências do gene 16S rRNA possuem limitada informação para determinação em nível de espécie dentro deste gênero. MLSA utilizando os genes 16S rRNA, 23S rRNA, *gyr*B e *rpo*B evidência que as espécies *M. amethystogenes, M. chromogenes, M. karnatakensis, M. parva, M. aerata, M. thermodiastatica* e *M. thermorosea* são espécies distintas da espécie *M. rosea*; diferentemente do proposto na literatura pro Miyadoh *et al.* (1990). No entanto *M. aerata* e *M. thermodiastatica* compartilharam elevada similaridade genética e provavelmente são a mesma espécie, assim como *M. indica* e *M. rosea*. Sugere-se assim os nomes *M. aerata* e *M. rosea* para as espécies acima, pois *M. aerata* e *M. rosea* foram descritas primeiramente que *M. thermodiastatica* e *M. indica*. As linhagens endofíticas da planta medicinal *V. divergens* pertencentes aos clusters *Microbispora* sp. 1, *Microbispora* sp. 2 e *Microbispora* sp. 3 são diferentes das espécies de *Microbispora* previamente descritas, sendo necessários maiores estudos para a descrição das mesmas como novas espécies. É proposto também a análise concatenada dos genes *gyr*B-*rpo*B como uma alternativa à técnica de hibridização de DNA para a identificação e analises filogenéticas dentro do gênero *Microbispora*, e o valor de 98,0% como linha de corte para determinar a relação em nível de espécie dentro deste gênero.

X. REFERÊNCIAS

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